Reactivity of Protein Histidines toward the Hydrated Electron

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ABSTRACT: The one electron reduction of protein histidines, identified by the appearance of an absorbance band near 360 nm, is observed upon reaction of the hydrated electron with δ-chymotrypsin, subtilisin BPN′, substilisin Carlsberg, thiosubtilisin, papain, and the inactive methyl methanethiosulfonate modified papain. With papain the yield of the imidazole–electron adduct is constant from pH 5.1 to pH 8.2. For the remaining proteins the absorbance yields are pH dependent with apparent p*K*s below 5 for δ-chymotrypsin, near 6.3 for the subtilisins, and 5.6 for the modified papain. There is little or no histidine reduction after the reaction of the hydrated electron with three chymotrypsin derivatives modified covalently at the active site, (phenylmethylsulfonyl)chymotrypsin, [67-*N*-methylhistidine]chymotrypsin, and anhydrochymotrypsin. Nor is there significant imidazole reduction seen with (phenylmethylsulfonyl)subtilisin BPN′ and with native subtilisin in the presence of the competitive inhibitor boric acid. We conclude that the sole or primary histidine reduced is that at the active site, with reduction occurring after migration of the electron from one or more initial attachment sites elsewhere on the protein.

On the basis of pulse radiolysis studies with small molecules, the hydrated electron (e_{aq})[1] should be capable of reacting with a variety of protein functional groups (Shafferman & Stein, 1975; Adams & Wardman, 1977; Klapper & Faraggi, 1979). Pulse radiolysis is a technique with which the hydrated electron and other radicals are produced in a short pulse (1 μs or less) of high-energy electron irradiation of aqueous solutions (Dorfman & Matheson, 1965). When the target molecules are proteins containing no prosthetic groups or metals, e_{aq} commonly reduces the disulfide bond and the imidazole side chain. Reductions at other functional groups on proteins most probably occur but are not well documented. The product of the disulfide reduction is the transient disulfide/one electron adduct ($RSSR^-$), easily detected by its strong absorption band centered in the region of 385–430 nm (the "410-nm" band). In small linear molecules, $RSSR^-$ decays rapidly to RS^- and RS·, both of which absorb weakly in the ultraviolet and have not been detected with proteins. $RSSR^-$ is also unstable in proteins, but unknown is whether the loss of the radical is through cleavage as occurs with small acyclic disulfides or disproportionation to form cystine plus cysteine as with cyclic radical disulfides such as lipoic acid. The one electron reduction product of the histidine side chain, the imidazole–electron adduct, absorbs in the region of 360 nm (the "360-nm" band). This unstable radical species decays to unknown products, but at rates considerably slower than those of the disulfide radical. Because the imidazolium ion reacts approximately 1000-fold faster with e_{aq} than does neutral imidazole, the reduction yield is pH dependent, from which dependence the histidine side chain p*K* can be determined in small peptides (Faraggi & Bettleheim, 1977).

In the attempt to measure protein histidine p*K*s with e_{aq} reduction, we (Faraggi et al., 1978a,b) obtained unexpected results: (i) imidazole reduction in α-chymotrypsin and trypsin at low pH but (ii) not in their respective precursors, chymotrypsinogen and trypsinogen, at any pH; (iii) no imidazole reduction in lysozyme, with its lone histidine located at the protein's surface and not at its active site (Blake et al., 1967); (iv) histidine reduction in RNase A with a measured histidine p*K* of 5.9, a value near to the p*K*s of the active site histidines in this enzyme (Roberts et al., 1969; Ruterjans & Witzel, 1969; Griffen et al., 1973; Markeley, 1975; Patel et al., 1975; Machuga & Klapper, 1977); (v) no reacting histidine associated with a p*K* near 7 in any of these enzymes. In explanation, we suggested that e_{aq} is specific to those histidines found at enzyme active sites. In order to verify this proposed specificity, we have extended our studies of the e_{aq} reaction to additional proteins and protein derivatives.

Materials and Methods

Pulse radiolysis experiments were performed in the linear accelerator facilities of the Ohio State University and Hebrew University, Jerusalem. The descriptions of these two machines may be found elsewhere (Felix et al., 1967; Klapper & Faraggi, 1983). The samples were dissolved in the buffer of choice (usually 10 mM phosphate and NaCl in 5 times distilled water with the pH adjusted as required) containing 0.1 M 2-methyl-2-propanol to scavenge the hydroxyl radical and deaerated with argon to prevent formation of superoxide by the e_{aq} reaction with oxygen. The quartz cell in which the samples were irradiated and the associated solution reservoir are shown schematically in Figure 1. When cooling of or additions to the stock solution were not required, we replaced the reservoir assembly with a syringe. Once in the reaction cell, the sample solution was exposed to a single pulse, approximately 500 ns in length, of ca. 3.5-MeV electrons to yield e_{aq} concentrations of 12–18 μM with an estimated reproduc-

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[1] Abbreviations: e_{aq}, the hydrated electron; RS^-, the thiolate group; RS·, the thiyl radical; NMe-chymotrypsin, α-chymotrypsin with *N*-methylhistidine-67 at the active site; PMS-chymotrypsin, α-chymotrypsin in which the active site serine at position 195 has been blocked with a phenylmethanesulfonyl group; anhydrochymotrypsin, α-chymotrypsin in which serine-195 has been converted to dehydroalanine; PMS-subtilisin, subtilisin BPN′ in which serine-221 has been converted to a cysteine; RNase A, pancreatic ribonuclease A; $RSSR^-$, the one electron reduced disulfide anion radical; Me_2SO, dimethyl sulfoxide; SMe-papain, papain in which the active site thiol has been reacted with methyl methanethiosulfonate.

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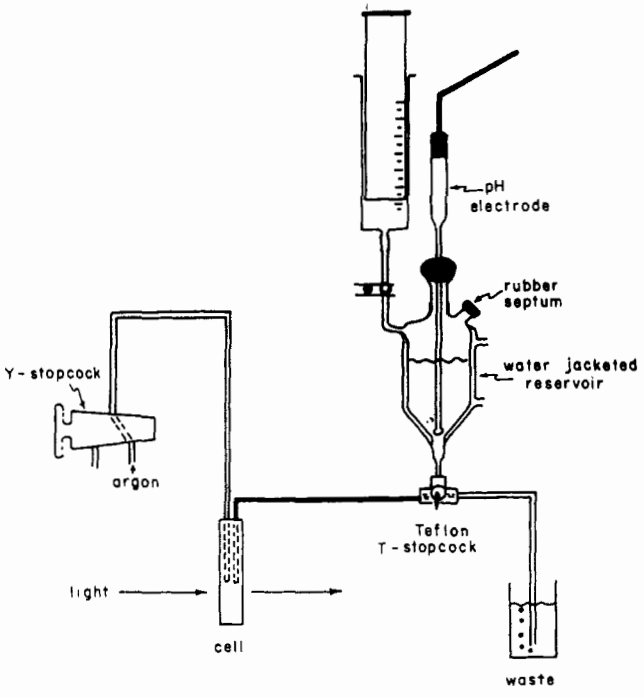

FIGURE 1: Diagram of reaction cell with attached reservoir. Spent solution is forced out of the reaction cell to the waste container by argon supplied through the Y stopcock on the left. The Y stopcock is then closed and the Teflon T stopcock opened to connect the reservoir to the cell. Protein solution is forced into the cell by pressure on the argon-filled syringe.

ibility of ±5%. Since the protein concentration was at least 10-fold higher than the e_{aq} concentration and since e_{aq} reacts with proteins at or near diffusion control, the probability of two electrons reacting with the same protein molecule is small (Klapper & Faraggi, 1979). Reactions that occurred after the pulse were monitored spectrophotometrically with the analyzing light path set at 90° to the electron beam. A sample was exposed to the electron beam only once. Spent solution was forced out of the cell with argon and fresh stock then introduced for the next determination.

Commercial 3 times crystallized α-chymotrypsin (Sigma) was purified over Sephadex G-25 (Pharmacia) to remove contaminating peptides (Wilcox, 1970). PMS-chymotrypsin (Wilcox, 1970), NMe-chymotrypsin (Ryan & Feeney, 1975), and anhydrochymotrypsin (Ako et al., 1972) were prepared by literature procedures from α-chymotrypsin. Chymotrypsinogen (Sigma) was converted to δ-chymotrypsin by the method of Wilcox (1970). The ovomucoid column used in the purification of the various chymotrypsin derivatives was prepared according to Maehler & Whitaker (1982). Chymotrypsin activity was measured by the *N-trans*-cinnamoylimidazole (Bender et al., 1966) and benzoyl-L-tyrosine ethyl ester (Wilcox, 1970) assays. Subtilisins BPN′ and Carlsberg were purified at 3–4 °C from commercial preparations by a modification of the Polgar & Bender (1967) procedure. Approximately 600 mg of protein dissolved in 20 mL of 10 mM phosphate/20 mM NaCl, pH 7.0, was applied to a 5 × 28 cm Sephadex C-50 column that had been preequilibrated with the same buffer. Three inactive protein peaks were eluted with this same buffer, and the NaCl concentration was then increased to 60 mM. Subtilisin with a specific activity of 1300 units/mg in the *p*-nitrophenyl acetate assay of Polgar & Bender (1969) was eluted next and frozen immediately for storage. Remaining protein with no subtilisin activity was removed from the column at higher ionic strength. On the day of its use, the subtilisin was thawed and quickly concentrated over an Amicon (Lexington, MA) PM-10 exclusion filter at 4 °C. The concentration was then adjusted (assuming $E^{1\%}_{280} = 1.17$; Ottesen & Svendsen, 1970) for pulse radiolysis studies. We kept the purified and frozen subtilisin for no longer than 4 days, since there is significant activity loss over longer time periods.

We prepared thiosubtilisin from subtilisin BPN′ with the slight modifications of published procedures (Polgar & Bender, 1967; Ottesen & Svendsen, 1970; Polgar, 1976). After reaction of commercial subtilisin BPN′ with phenylmethanesulfonyl fluoride, the crude PMS product was purified on a Sephadex C-50 column with the elution schedule described just above. The protein was concentrated and then dialyzed by ultrafiltration over an Amicon PM-10 filter. We used this PMS-subtilisin either for pulse radiolysis experiments or for conversion to thiosubtilisin. Crude thiosubtilisin was purified with a mercury column and stored as the Hg-blocked form. Immediately before its use, the blocked enzyme was incubated with a 100-fold excess of β-mercaptoethanol for 1 h at room temperature and applied to a 5 × 25 cm Sephadex G-25 column preequilibrated with an argon-deaerated buffer of choice. The protein was eluted with the same deoxygenated buffer while elution and collection reservoirs were kept under argon to minimize sulfhydryl oxidation. After elution, the protein was concentrated by ultrafiltration under argon and finally diluted to the desired experimental concentration with deoxygenated buffer.

Papain from commercial sources was purified with a variation of the mercury column methods (Sluyterman & Wijdenes, 1970, 1974; Sluyterman & de Graaf, 1972). Crystallized (2 times) papain (Sigma Chemical Co., St. Louis, MO) was dissolved (1 g by weight) in 900 mL of activating buffer (10 mM pyrophosphate, 10 mM Na_2SO_3, 20 mM EDTA, 20 mM 2-mercaptoethanol, and 2 mM dithiothreitol, pH 8.2) at room temperature for approximately 1 h. Solid ammonium sulfate was then added slowly to 65% saturation, and the resulting suspension placed in an ice–water bath for 40 min. The precipitate collected by centrifugation at 10000 rpm and 4 °C was then suspended in buffer A (50 mM acetic acid, 1 mM EDTA, 10 mM Na_2SO_3, 0.1 mM KCl, pH 5.0) 70% saturated with ammonium sulfate. After centrifugation, the precipitate was redissolved in buffer A (ca. 729 mg in 190 mL) and applied at a flow rate of 3.7 mL/min to a 2.5 × 12.5 cm Hg–Sepharose (2.9 μmol of mercury sites per mL of packed bed) column previously equilibrated with buffer A. The preparation of the Hg–Sepharose column and its use were taken from Sluyterman et al. (1970, 1972, 1974). After sample application, inactive protein was washed off with a solution of 50 mM sodium acetate–200 mM KCl, pH 5, to which ethanol (10% v/v) had been added. Papain was then eluted with 50 mM sodium acetate, 500 mM KCl, 2 mM mercuric acetate, and 10% ethanol, pH 5.0, yielding approximately 360 mg of Hg–papain on the basis of optical density at 280 nm (Bender et al., 1966). The protein solution volume was reduced by ultrafiltration through an Amicon PM-10 membrane. The eluting buffer contained ethanol rather than Me_2SO, since the latter destroys the Amicon membrane. The resulting suspension was stored at 4 °C.

Prior to use, the papain eluted from the Hg–Sepharose column was activated as follows. The desired amount of Hg–papain was transferred to a 15-mL glass centrifuge tube, and 2-mercaptoethanol and EDTA were added to 35 and 10 mM, respectively. After 1 h at room temperature, the solution was centrifuged, if not clear, and applied to a 5 × 24 cm Sephadex G-25 (Pharmacia) column preequilibrated with

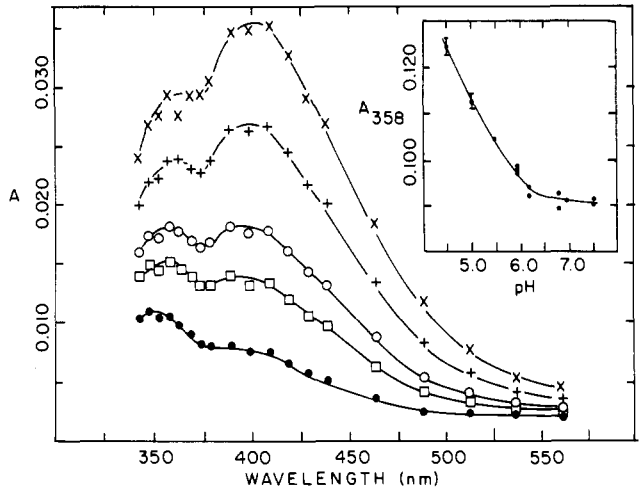

FIGURE 2: Spectra after reaction of hydrated electron with δ-chymotrypsin. Results obtained at room temperature 50 μs (×), 0.1 ms (+), 0.2 ms (○), 0.3 ms (□), and 0.8 ms (●) after the end of a 400-ns pulse of 3.5-MeV electrons. Concentrations of all components were as follows: 0.16 mM chymotrypsin, 15.4 μM e_{aq}, 10 mM phosphate, and 0.1 M 2-methyl-2-propanol, argon saturated, pH 4.6. (Inset) Absorbance yield at 358 nm 20 μs after the pulse as a function of pH. The solid line is drawn arbitrarily.

argon-flushed 0.01 M NaCl/0.01 M phosphate buffer at the desired pH. The protein was eluted with the same deoxygenated buffer while the system was kept under argon to minimize sufhydryl oxidation. The protein-containing peak was then concentrated under argon and finally diluted to the desired experimental concentrations (0.08–0.5 mM using $Ec_{280}^{1\%} = 24.7$; Bender et al., 1966) with deoxygenated buffer. When prepared this way, papain had 96% of the expected sulfhydryl content as measured with Ellman's reagent (Ellman, 1959). Papain activity was measured with benzoyl-L-arginine ethyl ester as substrate (Burke et al., 1974).

SMe-papain was kindly provided by Dr. Jules Shafer. All other chemicals were purchased commercially and used with no further purification, except for the 2-methyl-2-propanol, which was recrystaliized 5 times.

RESULTS

The spectra we observed after the reaction between protein and hydrated electron were constructed from individual kinetic experiments, each performed at a different wavelength and with fresh solution. Figure 2 is an example of the spectral changes seen with δ-chymotrypsin as reactant. (This enzyme contains five cystine disulfide bonds and two histidines, one at the active site.) There is a "410-nm" band characteristic of the $RSSR^-$ radical and a significant band near 360 nm due to histidine reduction. The relative magnitudes of the two bands change with time, consistent with the more rapid decay of $RSSR^-$ (Klapper & Faraggi, 1979). Plotted in the inset of Figure 2 is the pH dependence of the absorbance yield at 358 nm corrected for the competitive loss of e_{aq} to the hydronium ion. As the pH increases the yield decreases, leveling off above pH 6.0. Due to our inability to obtain reliable results below pH 4.5, an apparent pK cannot be assigned to this decrease but would have to be ≤5. The δ-chymotrypsin spectra and the pH dependence of the "360-nm" band are closely similar to our previous results with α-chymotrypsin and trypsin (Faraggi et al., 1978a).

In contrast, we find no prominent 360-nm band, at any pH, after the reaction of e_{aq} with PMS-, NMe-, and anhydrochymorypsin, three nonfunctional derivatives covalently modified at the enzyme's active site. The NMe-chymotrypsin spectra presented in Figure 3 are closely similar to the spectra

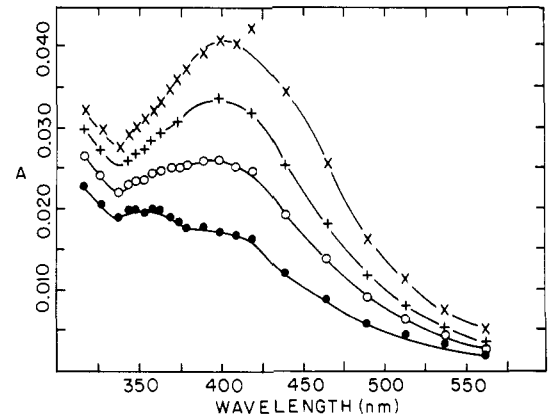

FIGURE 3: Spectra after reaction of hydrated electron with NMe-chymotrypsin. The conditions are similar to those in the legend of Figure 1: 0.14 mM protein, 13 μM e_{aq}, and pulse length 300 ns; 25 μs (×), 0.1 ms (+), 0.3 ms (○), and 0.8 ms (●) after the pulse.

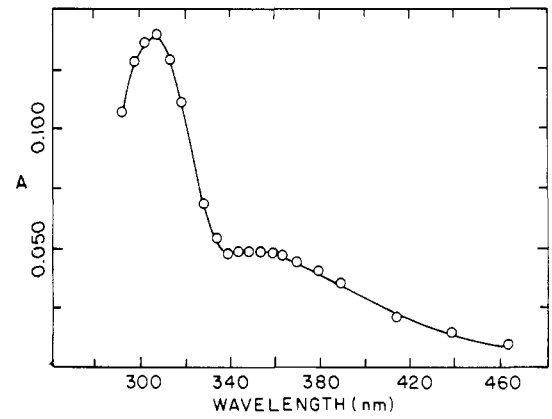

FIGURE 4: Spectra after reaction of hydrated electron with *N*-methylimidazole. Results obtained at room temperature 1 μs after the end of a 500-ns pulse of 3.5-MeV electrons. Concentrations of all components were as follows: 25 mM *N*-methylimidazole, 20 μM e_{aq}, 10 mM phosphate, and 0.1 M 2-methyl-2-propanol, argon saturated, pH 5.4.

obtained with the other two inactive derivatives. There is only a small shoulder in the region of 360 nm due either to residual histidine reactivity with the hydrated electron or to some other reduction product. (*N*-Methylimidazole reacts with e_{aq} to form a one-electron adduct with an absorption band near 360 nm, Figure 4.) We conclude that these three covalent modifications at the active site of chymotrypsin eliminate or greatly decrease the histidine reactivity with e_{aq}.

Subtilisins BPN′ and Carlsberg contain no cystines and six and five histidines, respectively, with one at the active site of each. The spectra we observed after the reaction of these proteins with the hydrated electron reflect the absence of cystine; the 360-nm band is prominent, and there is no major absorption band in the region around 410 nm (Figure 5). These results are close to those of Bisby and co-workers (Bisby et al., 1976). Similar spectra were obtained with thiosubtilisin, a derivative that retains the esterase but not the peptidase activity of the native enzyme (Ottesen & Svendsen, 1970). The yields of the 360-nm band exhibit pH dependencies that can be described as simple transitions with apparent pKs of 6.3 ± 0.6 for subtilisin BPN′, 6.3 ± 0.4 for subtilisin Carlsberg, and 6.4 ± 0.3 for thiosubtilisin. The thiosubtilisin results are presented in Figure 6. Thus, in contrast to chymotrypsin, a modification at the active site of subtilisin need not decrease or eliminate the reaction of e_{aq} with this protein's histidine residue(s). If, however, the active site serine is blocked with a phenylmethanesulfonyl group, as with chymotrypsin, there

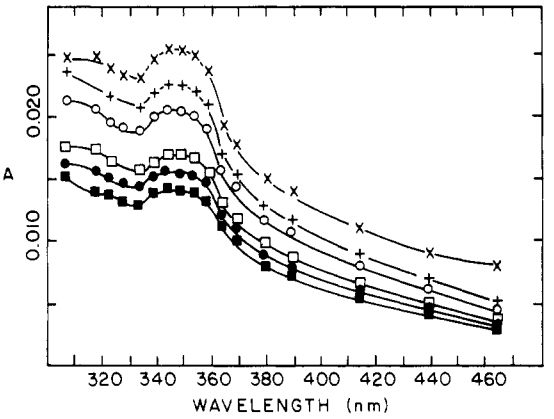

FIGURE 5: Spectra after reaction of hydrated electron with subtilisin BPN'. Results obtained at room temperature 10 μs (×), 50 μs (+), 0.1 ms (○), 0.3 ms (□), 0.5 ms (●), and 0.8 ms (■) after the end of a 400-ns pulse of ca. 3.5-MeV electrons. Concentrations of all components were as follows: 0.14 mM subtilisin, 11.7 μM e_{aq}, 10 mM phosphate, 60 mM NaCl, and 0.1 M 2-methyl-2-propanol, argon saturated, pH 5.6.

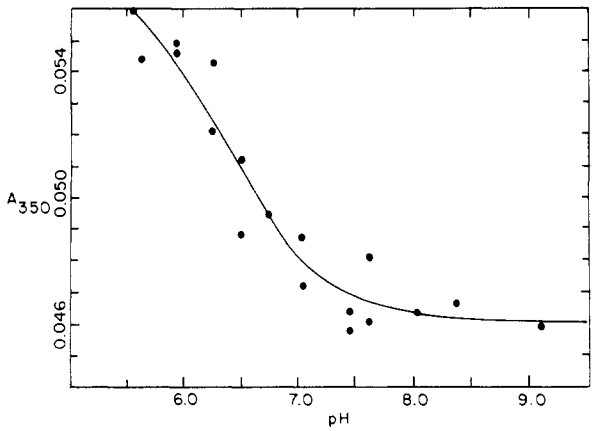

FIGURE 6: pH variation of absorbance at 350 nm 0.1 ms after reaction of hydrated electron with thiosubtilisin. Concentrations used were 0.3 mM thiosubtilisin and 25 μM 7 Otherwise, the conditions were identical with those given in the legend of Figure 5. The curve is calculated on the basis of the pK of 6.4 ± 0.3 obtained from a nonlinear least-squares fit of the data points.

is a marked decrease in the magnitude of the band near 360 nm. PMS substitution onto the subtilisin active site serine decreases the reactivity of one or more histidine side chains toward the hydrated electron. A noncovalent change at the subtilisin active site also appears to reduce the protein's histidine reactivity. The 360-nm absorbance yield is sharply decreased in the presence of 0.3 M boric acid, a competitive inhibitor (Robillard & Shulman, 1974; Jordan et al., 1982).

Papain contains three cystine disulfide bonds and one free sulfhydryl, which functions as a nucleophile in the active site (Lowe, 1976). It also contains two histidine residues, one of which functions as an acid/base group in the catalysis. Shafer and co-workers (Lewis et al., 1976, 1981; Johnson et al., 1981a,b) have proposed that this active site histidine has a pK of 8.6 in the succinylated protein but of 4.2 when the active site sulfhydryl is blocked by reaction with methyl methanethiosulfonate (SMe-papain).

Time-dependent papain spectra resulting from the reaction of protein and e_{aq} were constructed from individual kinetic experiments, each performed with fresh protein solution at different wavelengths. Figure 7 is an example of the spectra obtained with native papain at three different times. The major band near 410 nm is associated with the disulfide–electron adduct seen previously with papain (Clement et al., 1972). With this band is a shoulder near 380 nm, which becomes more pronounced at later times. At present, we assume that this shoulder also arises from a disulfide–electron adduct. Additionally, there is a band with apparent maximum near 355 nm. This is the absorption of the imidazole–electron adduct. As noted above with δ-chymotrypsin, the relative magnitudes of the "410-nm" and "360-nm" bands change with time, consistent with small disulfide and imidazole models (Klapper & Faraggi, 1979). The "360-nm" band is seen in papain spectra obtained over the pH range 5.1–8.2 (Figure 7), and over this pH range there is no change, within exper-

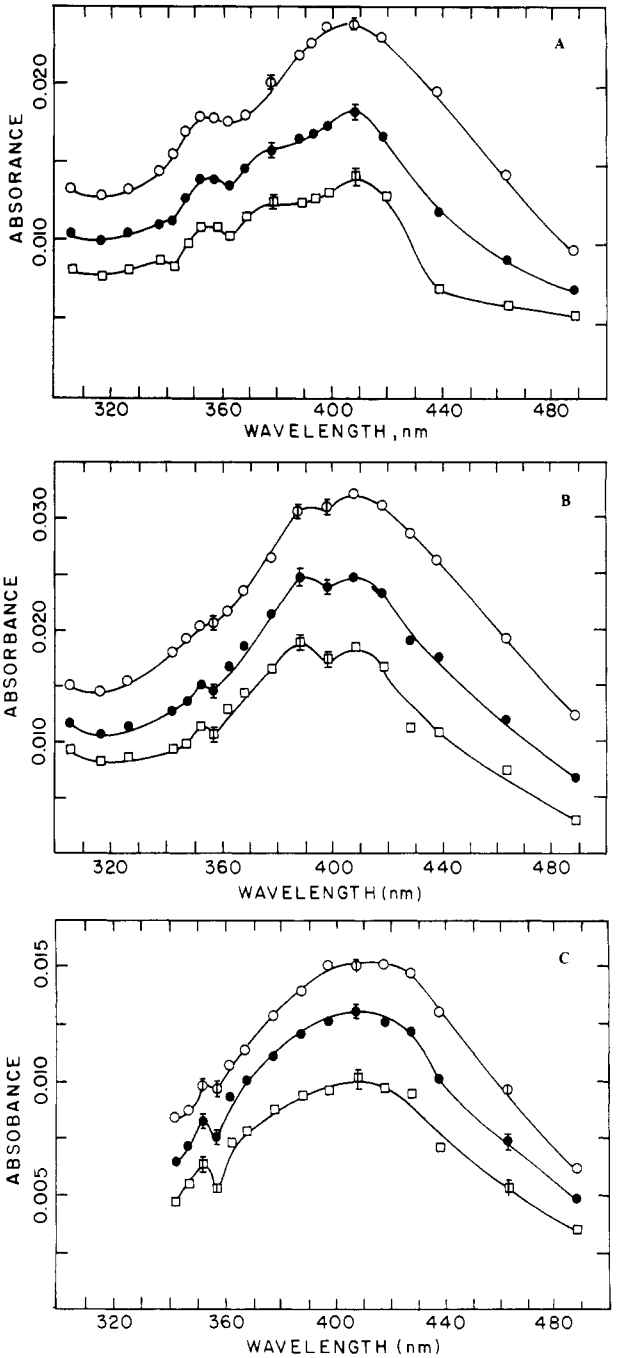

FIGURE 7: Spectra of papain following reaction with e_{aq}. All solutions contained 10 mM NaCl, 10 mM phosphate, and 0.1 M 2-methyl-2-propanol and were saturated with argon. (A) pH 5.6, 0.10 mM papain, 9.9 μM e_{aq}; (B) pH 7.1, 0.10 mM papain, 9.5 μM e_{aq}; (C) pH 7.9, 0.07 mM papain, 6.0 μM e_{aq}. The times at which the three spectra were recorded were, from top to bottom, 0.025, 0.3, and 1.8 ms after the end of the pulse. Each point is the average of more than one determination. A few representative standard deviations are presented in the form of error bars.

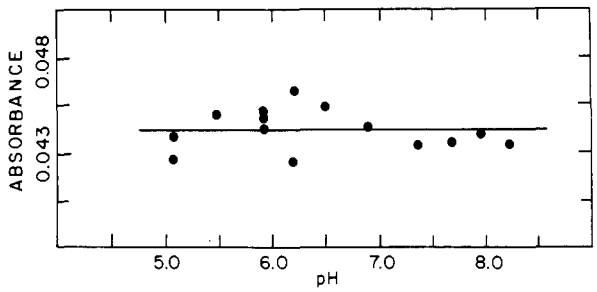

FIGURE 8: Papain reaction with e_{aq}: effect of pH on absorbance yield at 355 nm. The experimental conditions were similar to those of Figure 7 with the exception that the light path was 4 times longer in these experiments: 0.14 mM papain, 9 μM e_{aq}, absorbance measured 0.3 ms after the pulse.

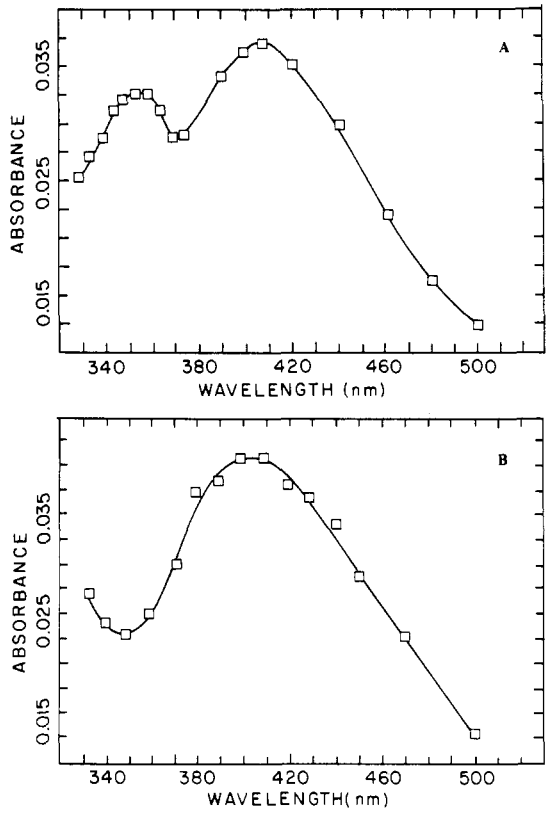

FIGURE 9: Spectra of SMe-papain following reaction with e_{aq}: (A) pH 5.0, 0.095 mM SMe-papain, 4.0 μM e_{aq}, 1 mM phosphate, 0.1 mM 2-methyl-2-propanol in argon-saturated solution at 8 μs after the end of the pulse. (B) pH 8.5, 4.9 μM e_{aq}, and other conditions identical with those of (A).

imental error, of the absorbance at 355 nm (Figure 8). We were unable to make measurements at higher pHs because of protein insolubility. Thus, native papain appears to have a histidine that remains reactive toward the hydrated electron up to a pH of 8.2. Since the reduction of unprotonated imidazole is too slow to measure by pulse radiolysis, we propose that this reactive histidine is protonated to at least pH 8.2.

We also studied the reaction of e_{eq} with SMe-papain. This derivative, in which the active site sulfhydryl has been blocked as the mixed disulfide $RSSCH_3$, also yields the histidine–electron adduct at low pH (Figure 9). At higher pH the "360-nm" band disappears, while the $RSSR^-$ band is observed at all pHs. The yield of the "360-nm" band as a function of pH is presented in Figure 10. The results can be analyzed in terms of a single titration curve with a pK of 5.6 ± 0.2. We do not know how significant is the difference between the two pKs obtained by NMR and pulse radiolysis: the measurements were taken at different ionic strengths, and the pulse radiolysis

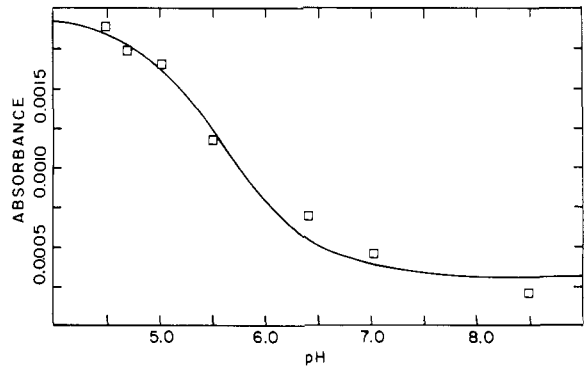

FIGURE 10: SMe-papain reaction with e_{aq}: effect of pH on yield of the "360-nm" band. The experimental conditions were identical with those of Figure 9. The yield of the "360-nm" band was estimated from the deconvolution of the observed spectra by assuming Gaussian curves in the plot of absorbance vs. wavenumber. The resultant absorbance at 355 nm was then normalized to account for small differences in the e_{aq} concentration between experiments.

result may reflect not only the imidazole pK but the reduction mechanism as well. However, the absence of a transition near pH 7—where the second histidine of the protein would be expected to titrate—together with the observed shift in pK between unmodified and –SH-blocked papain—paralleling the NMR results—suggest that the transition we observe with SMe-papain is due to the active site histidine. It is interesting that this imidazole still competes successfully for the electron even though there is a new disulfide bond in the active site.

Discussion

Since we believe that the experiments presented here serve to probe protei structure, it is appropriate for us to deal with some common misperceptions about the validity of protein pulse radiolysis studies; namely, the reactivity of the hydrated electron toward the numerous sites on a protein means a confusing multiplicity of simultaneous and consecutive radical reactions; radical reactions initiated by the hydrated electron damage the structural integrity of proteins; and hence, it is extremely difficult, if not impossible, to learn anything about the chemistry of native proteins with the technique of pulse radiolysis.

It is a generality (Klapper & Faraggi, 1979) that the reactions of proteins with e_{aq} occur at or near the diffusion-controlled limit, so that most or all first collisions yield reaction products. Consequently, when the protein to radical concentration ratio is sufficiently high, the probability of more than one e_{aq} reacting with a single protein molecule is negligible. Because of its large size and the resultant small diffusion coefficient, the protein becomes a temporary "island" to its one radical; and subsequent reactions, if sufficiently fast, proceed undisturbed by the events around them. Differences in relative reactivities of different protein sites toward e_{aq} must also be considered. Reaction of the hydrated electron at the protein surface should be fast relative to the time required for that radical to diffuse into the protein matrix, due both to the hydration shell and high charge density of e_{aq}. Thus, the initial reaction should occur on the protein surface and not at internal loci shielded from the aqueous environment. In turn, partitioning between subsequent potential reaction pathways should depend on the relative "concentrations" and intrinsic reactivities of the secondary reaction sites. Those with lower intrinsic reactivities would not compete effectively, unless they far outnumber the more reactive sites. This suppressive effect will tend to simplify the observed results. The selectivity in the measurement technique also simplifies the task of data interpretation. We, for example, monitor the appearance and

disappearance of transients and products by changes in optical absorbance, so that the detection of a reaction depends upon the spectral properties of the species involved. If there are processes that we cannot see, then the interpretation of those processes we do see becomes simpler, provided we stay within the time period over which potential protein to protein reactions contribute negligibly. (There most certainly are reactions we do not detect, since the measured yields of all products in any particular experiment infrequently exceed 60% of the amount of e_{aq} reacting with the protein.)

Nor need there be concern that the radical reactions involved in the pulse radiolysis experiment are so violent that the protein subject to these probes is alway denatured. First, this is most probably not true; and second, denaturation, were it to occur, is of no concern in the experiments reported here. We are interested in the amount of the radical adduct obtained during the microsecond or so of the reaction between hydrated electron and native protein, and not in the ultimate products derived from the reduced imidazole. Thus, we are probing the native structure before any gross structural change is possible and are "blind" to the denaturation that this probing may cause. In fact, were the reduction of a histidine to lead finally to denatured enzyme, the resultant activity loss would be difficult to determine. In the experiments with chymotrypsin, for example, the yield of reduced imidazole does not exceed 20% of the electrons adding to the protein. Realizing that only 5–10% of all protein molecules react with e_{aq} because of the high protein e_{aq} ratio used in our studies, it is clear that only 1–2% of the enzyme in the reaction could be denatured by the histidine reaction. Since we cannot measure so small a loss, the measurement of an enzyme's activity after a pulse radiolytic experiment tells us primarily whether the experimental conditions are appropriate; and it is only for this reason that we check an enzyme's activity.

We have previously suggested that the hydrated electron reduces protein histidine residues located at active sites in preference to those located elsewhere in the protein (Faraggi et al., 1978a,b). One piece of supporting evidence we cited at that time was the pH dependence of reduced histidine yields in trypsin and chymotrypsin, a dependence consistent with apparent histidine p*K*s below 4.5. Since a low p*K* had been suggested for the active site histidines of serine proteases (Hunkapiller et al., 1973; Koeppe & Stround, 1976; Markley & Porubcan, 1976), we concluded that the observed pH dependence reflected titration of the active site histidines in these two proteins. However, in concurrence with the earlier work of Robillard & Shulman (1972, 1974), more recent reports place the p*K* of the active site histidine in serine proteases near 7 (Bachovchin & Roberts, 1978; Bachovchin et al., 1981) and of bovine α-chymotrypsin near 6 (Markley et al., 1978). Thus, the interpretation of the low p*K* value extracted from the pulse radiolysis data has become problematic.[2]

To verify that the primary α-chymotrypsin histidine residue reactive toward e_{aq} is at the active site, we studied three enzyme derivatives modified covalently at that locus: the *N*-methylhistidine-57 derivative, which retains a very low residual activity, and the inactive [(phenylmethayl)sulfonyl]serine-195 and anhydrochymotrypsin derivatives. In the e_{aq} product absorption spectrum of each derivative, the absorbance band near 360 nm, which is the signature of reduced histidine, is almost entirely eliminated; there is little or no reaction of e_{aq} with the histidines of these three derivatives. These results extend our earlier observations that the inactive zymogens of trypsin and chymotrypsin also show little histidine reactivity toward the hydrated electron. Thus, in all the modifications of these two proteins that we have investigated there is (i) near total or total loss of enzyme activity and (ii) markedly decreased histidine reactivity toward e_{aq}. We conclude that the e_{aq}-reactive histidine in chymotrypsin and trypsin is at the active site.

This conclusion leaves us with an apparent discrepancy between the active site histidine p*K*s measured by pulse radiolysis, <5, and those based on NMR data, 6–7. In support of the low α-chymotrypsin p*K* measured with pulse radiolysis, we here report a similar pH dependence in the hydrated electron reaction with δ-chymotrypsin. α-Chymotrypsin is known to dimerize (Aune & Timasheff, 1971; Aune et al., 1971) at these low pHs, and the extent of dimerization increases as the pH is lowered to reach a maximum near pH 4.4 (Miller et al., 1971). But the yield of the imidazole–electron adduct has a similar magnitude and pH dependence in the case of δ-chymotrypsin, which has a lower tendency to dimerize (Miller et al., 1971). Thus, dimerization does not appear to be necessary for histidine reduction in α-chymotrypsin. The following are possible arguments in explanation of the apparent difference between NMR and pulse radiolysis results.

First, the greater reactivity of e_{aq} toward protonated imidazolium as compared with unprotonated imidazole must, at least in part, be the consequence of an electrostatic promotion by the imidazolium charge. Thus, the apparent chymotrypsin and trypsin histidine p*K*s measured by pulse radiolysis may reflect the overall charge distribution in the region of the imidazole ring rather than just the physical protonation of that ring. We could then argue that there is insufficient positive charge around the active site imidazole for a *successful competition* with other groups on the protein (such as the disulfide bond) that also react with e_{aq}, unless the neighboring aspartic acid-102 side chain carboxyl is also protonated. This would result in a macro p*K* lower than the micro p*K* associated with protonation of the imidazole ring. Second, at present the mechanism with which the hydrated electron appears to select the active site histidine is unknown. Within this mechanism may lie the cause of the apparently low p*K*. Finally, the NMR titrations must also yield a macro p*K* that may well differ from the intrinsic acid dissociation constant of the histidine-67 imidazolium group.

The subtilisin results show that a covalent alteration at the enzyme active site need not lead to loss of histidine reduction by e_{aq}. There is histidine reduction in both subtilisin and thiosubtilisin, the latter of which retains the esterase but not the peptidase activity of the native protein (Ottesen & Svendsen, 1970). The apparent p*K* associated with these reactions is approximately 6.3 for subtilisin BPN′, subtilisin Carlsberg, and thiosubtilisin. While the p*K* of the active site histidine has not yet been assigned, four of the six histidine resonances located in NMR spectra are associated with p*K*s between 5.4 and 6.5 in PMS-subtilisin (Omar et al., 1979) and 7 ± 0.5 in subtilisin (Jourdan et al., 1982).

When we block the active site serine of subtilisin by reaction with phenylmethanesulfonyl fluoride or when we mix subtilisin with the competitive inhibitor borate, then there is a loss of

[2] It should be noted that the p*K*s based on NMR titrations may also be problematic. Markley et al. (1978) observed two transitions in the NMR titration of the α-chymotrypsin active site histidine with p*K*s at 2.8 and 6.1. Their assignment of the higher p*K* to imidazole protonation was argumental. On the basis of sedimentation data, Aune & Timasheff (1971) have suggested a p*K* of 5.0 for the active site histidine in monomeric α-chymotrypsin and 6.2 in the dimeric species. Fersht & Renard (1974) suggested a p*K* between 6.2 and 6.5 on the basis of proton release during substrate binding to α-chymotrypsin.

Table I: Observed Histidine Reduction

	360-nm band[a]	apparent pK_a
trypsin	+	<4.5
trypsinogen	−	
α-chymotrypsin	+	<4.5
δ-chymotrypsin	+	<5
chymotrypsinogen	−	
PMS-chymotrypsin	−	
NMe-chymotrypsin	−	
anhydrochymotrypsin	−	
subtilisin BPN′	+	6.3 ± 0.6
subtilisin Carlsberg	+	6.3 ± 0.4
thiosubtilisin	+	6.4 ± 0.3
PMS-subtilisin	−	
subtilisin + borate	−	
papain	+	>8.2
SMe-papain	+	5.6 ± 0.2
ribonuclease	+	~5.9
$\alpha_{s,1}$-casein	+	
lysozyme	−	
lactalbumin	−	

[a] The results have been collected from Faraggi et al. (1978a,b) and this paper.

histidine reactivity. We conclude from these results that the histidine "visualized" by reaction with e_{aq} is at the subtilisin active site.

The pK_a values assigned to the active site histidine of papain and SMe-papain are 8.6 (succinylated papain; Lewis et al., 1981) and 4.2 (Johnson et al., 1981a). The pulse radiolysis results suggest pK_a of >8.2 and 5.6, respectively. We do not know how significant is the difference in NMR and pulse radiolysis results for SMe-papain, but the pK trend is the same for both sets of data. Hence, we conclude that the pulse radiolysis experiments also "see" the active site histidine of papain, although this is the first example we have of a protein in which an inactive enzyme derivative also yields the imidazole–electron adduct (a summary of all our results to date is presented in Table I).

Whether our observations of differences in the reactivity of active site and non-active-site histidines toward e_{aq} are general over a larger number of proteins will be determined as more data are collected. Irrespective of future results, it is surprising the e_{aq} with a redox potential of ≤−2.7 V (Hart & Anbar, 1970) should appear to have such a high specificity; not only do we detect just the active site histidine, but also covalent or noncovalent modifications at the active site modulate that histidine reaction. We feel that imidazole accessibility to e_{aq} cannot explain this apparent specificity. First, the single histidine of lysozyme, which is solvent accessible on the basis of X-ray results (Blake et al., 1967) but is not at the enzyme's active site, does not yield the imidazole–electron adduct (Faraggi et al., 1978b). Second, it does not seem likely that the histidine accessibilities in the active sites of chymotrypsin, anhydrochymotrypsin, and chymotrypsinogen should differ greatly; yet the first yields a "360-nm" band, and the latter two do not.

We have suggested elsewhere (Klapper & Faraggi, 1979) that the primary sites of e_{aq} reaction in proteins may not be the histidines or disulfides, the reductions of which we and others observe spectrally. Since the hydrated electron is embedded within a water shell, it is reasonable to argue that the initial interaction of e_{aq} and protein must at the very least involve electron desolvation at the protein surface. We believe that this initial event is followed by intramolecular electron transfer to the histidine. Not only is there circumstantial evidence for such transfers in proteins (Klapper & Faraggi, 1979), but more recently there have been reports of long-distance electron transfers in model systems [e.g., Prutz et al. (1981), Guar et al. (1983), Calcaterra et al. (1983), and Isied & Vassilian (1984)] and in proteins (Prutz et al., 1983; Winkler et al., 1982; Isied et al. 1984;, Faraggi & Klapper, 1984; Faraggi et al., 1985). In two of these citations a 15-Å intramolecular transfer in cytochrome c is reported to occur with a rate constant of 20–50 s^{-1}. Were the reduction of an imidazole to involve intramolecular electron migration, then the rate and yield of radical formation could depend both on the driving force of the reaction, the redox pair potential, and on the structural reorganization energy required by such a transfer [e.g., Buhks et al. (1981), Hopfield (1974), and DeVault (1980)]. Thus, the apparent specificity of the histidines at active enzyme sites may reflect structural peculiarities that lower the potential energy of the imidazole and/or its electron adduct at these sites. The structure of globular proteins forces a nonuniform distribution of charged and polar groups in and around a low dielectric, apolar core. Introducing an electron of sufficient energy into a protein and then tracing its migration therein may serve to probe experimentally the resultant potential surface. The histidine results we have obtained suggest that there may be a unique charge distribution associated with histidines at active sites. It is intriguing to speculate whether proteins utilize some such unique distribution for catalytic purposes.

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