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Selective Oxidation of Methionine Residues in Proteins†

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ABSTRACT: Methionine residues in peptides and proteins were oxidized to methionine sulfoxides by mild oxidizing reagents such as chloramine-T and *N*-chlorosuccinimide at neutral and slightly alkaline pH. With chloramine-T cysteine was also oxidized to cystine but no other amino acid was modified; with *N*-chlorosuccinimide tryptophans were oxidized as well. In peptides and denatured proteins all methionine residues were quantitatively oxidized, while in native proteins only exposed methionine residues could be modified. Extent of oxidation of methionine residues was determined by quantitative modification of the unoxidized methionine residues with cyanogen bromide (while methionine sulfoxide residues remained intact), followed by acid hydrolysis and amino acid analysis. Methionine was determined as homoserine and methionine sulfoxide was reduced back to methionine. Sites of oxidation were identified in a similar way by cleaving the unoxidized methionyl peptide bonds with cyanogen bromide, followed by quantitative end-group analysis of the new amino-terminal amino acids (by an automatic sequencer).

The thioether groups of methionine side chains in proteins are weak nucleophiles, and, like other hydrophobic residues, usually have little access to the aqueous environment. In contrast with other nucleophilic residues in proteins, they resist protonation from pH 1 to 14 and can therefore be selectively modified under acidic conditions. Alkylation with halo acids and oxidation with hydrogen peroxide were extensively used to modify methionine side chains in proteins.

Hydrogen peroxide, under suitable, relatively mild conditions, will modify several functional groups in proteins, such as thioethers, indoles, sulfhydryls, disulfides, imidazoles, and phenols. Under acidic conditions the primary reaction is oxidation of methionines (Toennies and Callan, 1939; Caldwell and Tappel, 1964), while under neutral and slightly alkaline conditions, tryptophan (Hachimori et al., 1964) and other functional groups are also modified (Means and Feeney, 1971), especially in the presence of metal ions, halide ions, organic acids, or ethers. Most studies in which a specific reaction with methionine residues has been documented have been carried out at pH below 4, such as in pancreatic ribonuclease (Neumann et al., 1962), α-chymotrypsin (Koshland et al., 1962; Schachter et al., 1963; Weiner et al., 1966), Kunitz trypsin inhibitor (Kassell, 1964), adrenocorticortropic and parathyroid hormones (Dedman et al., 1961; Tashjian et al., 1964), and chymotrypsinogen (Wasi and Hofmann, 1973). Exceptions were the oxidations of glucose oxidase at pH 5.8 (Kleppe, 1966), subtilisin at pH 8.8 (Stauffer and Etson, 1969), and bovine growth hormone at pH 8.5 (Glaser and Li, 1974).

In addition to hydrogen peroxide, a number of other reagents have been used for oxidizing methionine residues in proteins. Periodate has been shown (Clamp and Hough, 1965) to attack cysteine, cystine, methionine, tryptophan, tyrosine, histidine and N-terminal serine and threonine. It has, however, been used for selective oxidation of methionine residues in α-chymotrypsin (Knowles 1965) and apomyoglobin (Atassi, 1967) under selected critical conditions at pH 5 and 0°. Similarly azide, under acidic conditions (hydrazoic acid), was shown to oxidize methionine to methionine sulfoximine (Whitehead and Bently, 1952; Brill and Weinryb, 1967).

Iodination of proteins at neutral and slightly alkaline pH causes iodination of histidine, tyrosine, and tryptophan, as

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well as oxidation of methionine to methionine sulfoxide (Koshland et al., 1963; Filmer and Koshlnnd, 1964).

Photooxidation was also used for oxidation of methionine residues, but was found to have poor selectivity (Spikes and Straight, 1967; Foote, 1968). Other oxidants such as *N*-bromosuccinimide (NBS)[1] or 2,4,6-tribromocyclohexadienone (Burstein and Patchornik, 1972) and tetranitromethane (Nilsson and Lindskog, 1967) were even less selective.

While studying iodination of flagellin (with iodine and chloramine-T) Parish and Stanley (1972) noticed that methionine residues of this protein could be oxidized with chloramine-T. The selectivity of this reagent was attributed to the absence of other oxidizable amino acids such as cystine, tryptophan, and histidine.

The present paper describes a method for selective oxidation of methionine residues in non-SH proteins under neutral and slightly alkaline conditions using chloramine-T and *N*-chlorosuccinimide (NCS). Analytical techniques for locating the modified residues were also developed.

Experimental Section

Materials

Bovine α-lactalbumin was isolated from raw skimmed milk by a modification of the method of Aschaffenburg and Drewry (1957) as described by Castellino and Hill (1970). Bovine α-chymotrypsin, bovine pancreatic ribonuclease, and hen egg-white lysozyme were purchased from Worthington Biochemical Corp. 8CM-Ribonuclease was prepared as described earlier (Sperling et al., 1969). 8CM-α-Lactalbumin was prepared according to Shechter et al. (1973).

Cyanogen bromide and chloramine-T were Fluka products; *N*-chlorosuccinimide was purchased from Pfaltz and Bauer, Inc., dithioerythritol was from Calbiochem; urea and *N*-bromosuccinimide were from BDH Chemicals; Trizma base and glutathione were from Sigma; 5,5′-dithiobis(2-nitrobenzoic acid) was from Aldrich; Sephadex G-25 and G-100 were from Pharmacia. The standard calibration mixture of amino acids Type I (which does not contain cysteine) was purchased from Spinco-Beckman and an equimolar amount of tryptophan was added to it.

Methods

Spectrophotometric measurements were performed with a Beckman Acta V spectrophotometer. Quartz cells of 1-cm light path and rectangular quartz tandem cells of 0.437-cm light path per chamber were used.

Sequenator analyses were performed with a Beckman sequencer, Model 890C, by the methods of Edman and Begg (1967), as modified by Hermodson et al. (1972). The sequenator reagents were products of Beckman and Pierce.

Amino acid analyses were performed with a Beckman automatic amino acid analyzer, Model 120C, after hydrolysis in 6 *N* HCl for 22 hr at 110° in the presence of dithioerythritol. Analyses of tryptophan residues in polypeptides and proteins were performed according to Liu coffone et al. (1968), as described earlier (Shechter et al., 1972, 1974). Free tryptophan was determined by automatic amino acid analysis using a 15-cm column at pH 5.25; elution time was 45 min (lysine was eluted after 60 min).

Determination of methionine sulfoxide in the presence of methionine was performed as follows: the oxidized protein was treated with cyanogen bromide in 80% formic acid according to Gross (1967). The methionine residues were thus converted to homoserine (lactone) residues, while the methionine sulfoxides remained intact. The sample was then lyophilized and hydrolyzed in 6 *N* HCl at 110° for 24 hr in the presence of dithioerythritol, reducing back methionine sulfoxide to methionine (in over 95% yield). The sample was subjected to automatic amino acid analysis; homoserine and its lactone represented the methionine content of the original sample, while methionine represented its methionine sulfoxide content.

Oxidation of Free Amino Acids with NBS, NCS, or Chloramine-T. To a standard solution of free amino acids (see Materials), an equimolar amount of L-tryptophan was added. The solution was evaporated to dryness at room temperature under vacuum and redissolved in the appropriate buffer. The 0.5-ml final volume of reaction mixture contained: 0.2 μmol of each amino acid, 0.1 *M* buffer (sodium citrate (pH 2.2) or Tris-HCl (pH 8.5)) and increasing amounts of oxidant. The mixture was allowed to react for 20 min at room temperature, whereupon *N*-acetylmethionine was added to destroy the excess reagent. The pH of the reaction mixture was adjusted to pH 2.0, and the sample subjected to automatic amino acid analysis.

Sulfhydryl groups were determined quantitatively according to Ellman (1959).

Glutathione (GSH) and oxidized glutathione (GSSG) were determined by the automatic amino acid analyzer; elution times were: GSH, 40 min; GSSG, 47 min (long column, pH 3.25).

Oxidation of Methionine Residues in Polypeptides and Proteins. The protein (2–3 mg) was dissolved in 1.0 ml of 0.1 *M* Tris-HCl buffer (pH 8.5) and an aqueous solution of the oxidant (10 μmol/ml) was added to it. The reaction was allowed to proceed for 20 min at room temperature, and the mixture was then subjected to gel filtration on a Sephadex G-25 column (1 × 30 cm) and eluted with 0.07 *M* ammonium bicarbonate. The fractions corresponding to the protein peak were combined, the absorption spectrum was measured, and amino acid composition was determined.

Oxidation of Glutathione (GSH) with Chloramine-T. To a solution of glutathione (1 μmol/ml) in 0.1 *M* Tris-HCl (pH 7.5) increasing quantities of methionine (0–15 μmol) were added and the volume was adjusted to 0.5 ml with the same buffer solution. Chloramine-T (1 μmol) was then added and after 15 min, at room temperature, samples were withdrawn for determination of sulfhydryl content (Ellman, 1959) and for amino acid analyses.

Results

Oxidation of Amino Acids with NBS. The sensitivity of free amino acids other than cysteine to oxidation with NBS was investigated; the results of this investigation are presented in Figures 1 and 2; 12 equiv of NBS was needed to completely modify methionine, tryptophan, cystine, tyrosine, and histidine at pH 2.2 (Figure 1), while other amino acids (see Materials) remained intact. Methionine was most sensitive to oxidation; it was first oxidized to methionine

[1] Abbreviations used are: NBS, *N*-bromosuccinimide; NCS, *N*-chlorosuccinimide; 8CM-ribonuclease, ribonuclease derivative in which all four disulfide bonds were reduced and carboxymethylated; 8CM-α-lactalbumin, α-lactalbumin derivative in which all four disulfide bonds were reduced and carboxymethylated; chloramine-T, *N*-chloro-*p*-toluenesulfonamide; BNPS-skatole, 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); GSH, glutathione; GSSG, oxidized glutathione; Pth, phenylthiohydantoin.

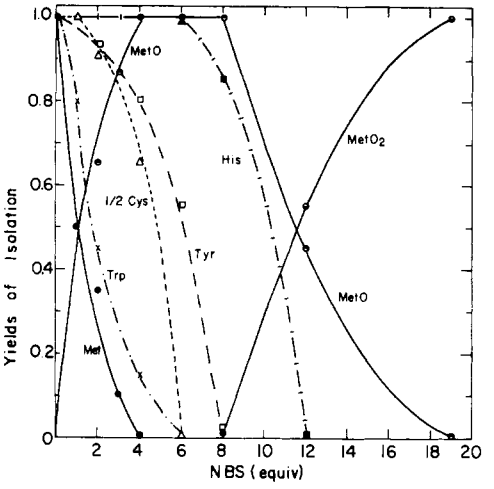

FIGURE 1: Competition between different amino acids for NBS. A mixture of 18 amino acids, 0.2 μmol of each (see Methods), was treated with increasing amounts of NBS in 0.1 *M* sodium citrate (pH 2.2) for 20 min at room temperature. Yields of isolation of free amino acids were determined by automatic amino acid analysis: methionine (●), methionine sulfoxide (⊖), tryptophan (X), cystine (△), tyrosine (□), histidine (■), methionine sulfone (⊖).

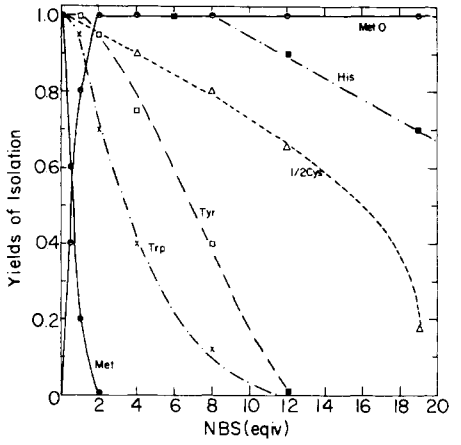

FIGURE 2: Competition between different amino acids for NBS in 0.1 *M* Tris-HCl buffer (pH 8.5). Conditions are the same as in Figure 1.

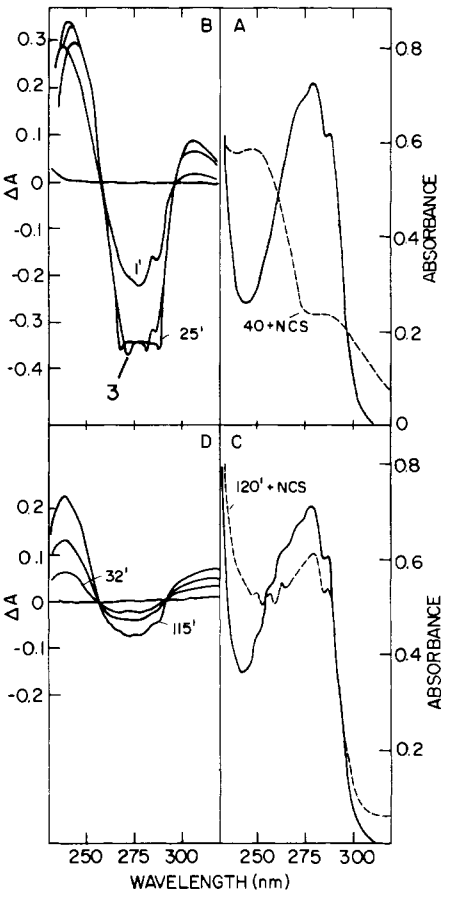

FIGURE 3: Spectrophotometric monitoring of the reaction of Ac-Trp-Gly with *N*-chlorosuccinimide. The reactions were performed at 25°. To 1.0 ml of the peptide solution (1.3×10^{-4} *M*) 10 equiv of NCS (in water) was added. (A) Absorption spectrum before (—) and after (- - -) incubation with NCS for 40 min in 0.1 *M* sodium acetate (pH 4.0). (B) Difference spectra of the reaction mixture (from 3A) at indicated times. (C) Absorption spectra before (—) and after (- - -) incubation with NCS for 120 min in 0.1 *M* Tris-HCl buffer (pH 8.5). (D) Difference spectra of the reaction mixture (from 3C) at indicated times.

sulfoxide, and only at higher concentrations of NBS was it further oxidized to methionine sulfone.

At pH 8.5 (Figure 2), the rates and order of oxidation were altered. Methionine was oxidized very rapidly to methionine sulfoxide, tryptophan and tyrosine were next oxidized, and only at higher concentrations of NBS could histidine be transformed. Methionine sulfoxide could not be oxidized to methionine sulfone unless histidine was first completely brominated. The rates of oxidation of tryptophan, tyrosine, cystine, and histidine at pH 8.5 were slower than at pH 2.2, whereas that of methionine was hardly affected by change in pH. By limiting excess NBS, we could selectively oxidize methionine to methionine sulfoxide at pH 8.5.

Oxidation of Amino Acids with NCS. When NCS was used to oxidize a mixture of amino acids, the results differed from those with NBS. Both at pH 2.2 and 8.5, only methionine and tryptophan were oxidized, whereas all other amino acids remained intact. Using 1 equiv of NCS methionine was oxidized to methionine sulfoxide at both pH's. No further oxidation of methionine sulfoxide was observed even when 50 equiv of NCS was used. The NCS oxidation was also carried out in a pH-Stat (absence of buffer) over the pH range 6.5–8.5, and in aqueous buffered solutions of 0.2 *M* sodium phosphate (pH 6.5–7.5) and Tris-HCl (pH 7.5–8.5). In all these experiments using 20 equiv of NCS, only methionine and tryptophan were oxidized.

Oxidation of Amino Acids with Chloramine-T. When chloramine-T was used as an oxidant, still other results were obtained. At pH 2.2, only methionine and tryptophan were oxidized (as with NCS). At pH 8.5 the reaction was even more specific. Methionine was oxidized to methionine sulfoxide, while tryptophan and all other amino acids remained intact.

Oxidation of Ac-Trp-Gly by NCS and Chloramine-T. The ultraviolet absorption spectra of Ac-Trp-Gly before and after reaction with NCS and chloramine-T are displayed in Figures 3 and 4. NCS at pH 4.0 (Figure 3A and B) rapidly oxidized the indole moiety of this peptide, as evidenced by a marked decrease in the absorbance at 280 nm (Patchornik et al., 1960). The oxidation was completed within 3 min using a tenfold excess of NCS. NCS oxidized the peptide at pH 8.5 as well, but the rate of oxidation was much slower than at pH 4.0 (Figure 3C and D). Using a tenfold excess of NCS only 5% of the peptide was oxidized in 10 min.

Chloramine-T at pH 4.0 (Figure 4A and B) oxidized Ac-Trp-Gly very rapidly, but in contrast to NCS, it did not oxi-

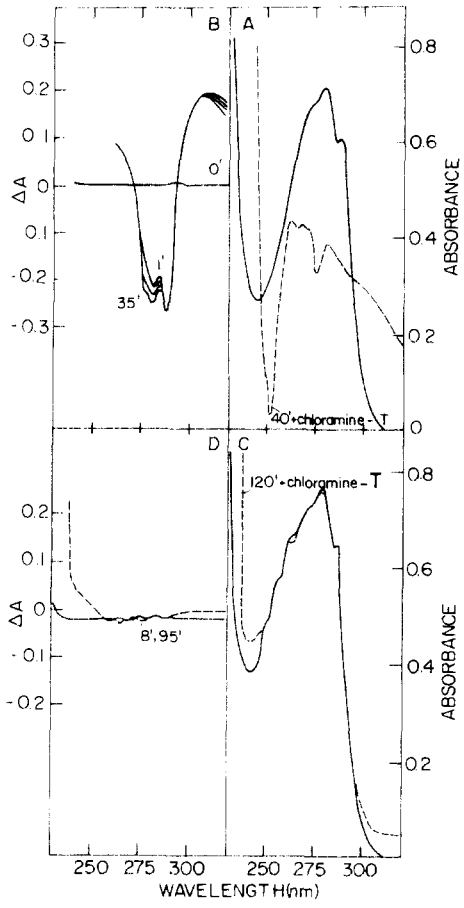

FIGURE 4: Spectrophotometric monitoring of the reaction of Ac-Trp-Gly with chloramine-T. Reaction conditions were identical with those of Figure 3. (A) Absorption spectrum before (—) and after (- - -) incubation with chloramine-T for 40 min in 0.1 *M* sodium acetate (pH 4.0). (B) Difference spectra of the reaction mixture (4A) at indicated times. (C) Absorption spectra before (—) and after (- - -) incubation with chloramine-T for 120 min in 0.1 *M* Tris-HCl (pH 8.5). (D) Difference spectra of the reaction mixture (4C) at indicated times.

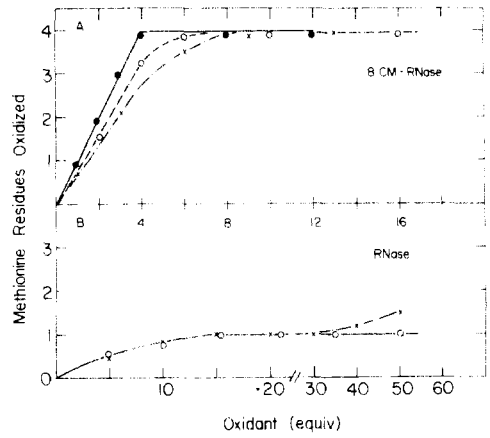

FIGURE 5: Oxidation of methionine residues in ribonuclease derivatives with increasing quantities of oxidants. The protein (3 mg) was dissolved in 1.0 ml of 0.1 *M* Tris-HCl (pH 8.5) and an aqueous solution of the oxidant (10 μmol/ml) was added to it. After 20 min the protein was purified by gel filtration, and analyzed for its Met and MetO content as described under Methods. (A) 8CM-ribonuclease; (B) ribonuclease. Oxidants: NBS (●), NCS (○), chloramine-T (x).

dize the tryptophan residue at pH 8.5 (Figure 4C and D).

Oxidation of Glutathione (GSH) with Chloramine-T. The oxidation of free sulfhydryl groups with chloramine-T was studied using GSH as a model compound. We have found that chloramine-T oxidizes GSH to GSSG (at pH 7.5). No further oxidation of GSSG was detected when excess chloramine-T (up to 20 equiv) was used, as was determined by amino acid analysis of GSSG and by the presence of the expected quantity of cystine and lack of cysteic acid from hydrolysates of the GSSG thus formed.

In a series of competition experiments, mixtures of GSH and methionine were oxidized (see Methods). It was found that 4.5 equiv of methionine was needed to cause 50% inhibition of oxidation of GSH by chloramine-T at pH 7.5.

Modification of Amino Acid Residues in Polypeptides. 8CM-Ribonuclease and 8CM-α-lactalbumin were treated with increasing quantities of NBS, NCS, and chloramine-T at pH 8.5, for 15 min at room temperature. Excess reagent was removed by gel filtration, and the modified protein was treated with cyanogen bromide in 80% formic acid for 24 hr at room temperature. Excess reagent and solvent were removed by freeze-drying, the protein was hydrolyzed, and the amino acids were analyzed.

With 8CM-ribonuclease (Figure 5A), 4 equiv of NBS oxidized all four methionine residues in the protein to methionine sulfoxides. No other residue was modified. With increasing quantities of NBS, tyrosine residues were also brominated. (The protein does not contain tryptophan residues.) When NCS was used as an oxidant, 6 equiv was needed to oxidize all four methionine residues to methionine sulfoxide. No other amino acid was modified, even when 16 equiv of NCS was used. With chloramine-T as the oxidant, 8 equiv was needed to oxidize all four methionines. No other residue was modified even in the presence of 50 equiv of this oxidant.

With 8CM-α-lactalbumin, the results were similar. One equivalent of NBS was required for the oxidation of the single methionine residue of this protein, whereas 4 equiv of NCS and 6 equiv of chloramine-T were needed for the same purpose. Again, no other amino acid was modified, as determined either by spectroscopic measurements or amino acid analysis following acid hydrolysis. However, when larger excesses of two of these oxidants were used, other amino acids were oxidized as well. With 20 equiv of NBS, tryptophans and tyrosines were also oxidized, with 20 equiv of NCS only tryptophans. With 20–50 equiv of chloramine-T, however, only methionine was oxidized.

An additional proof of the oxidation of the single methionine residue in 8CM-α-lactalbumin was obtained by treating the protein with cyanogen bromide. Unoxidized 8CM-α-lactalbumin was cleaved with cyanogen bromide in 80% formic acid, and the cleavage products were separated by gel filtration on a Sephadex G-100 column. The elution pattern of this column is shown in Figure 6A. Peak CB_0 (fractions 29–33) represents the uncleaved protein, peak CB_1 (fractions 36–45) residues 1–90 of 8CM-α-lactalbumin, and peak CB_2 (fractions 52–60) represents residues 91–123 (Shechter et al., 1973). In another experiment, 8CM-α-lactalbumin was first oxidized with 10 equiv of chloramine-T at pH 8.5 prior to treatment with cyanogen bromide in 80% formic acid for 24 hr at room temperature. The reaction mixture was freeze-dried and the residue loaded onto a Sephadex G-100 column. The elution pattern (Figure 6B) revealed the presence of only one peak, CB_0, indicating that no cleavage had occurred.

Oxidation of Met^{192} in α-Chymotrypsin. The above-described experiments indicated that methionine residues in

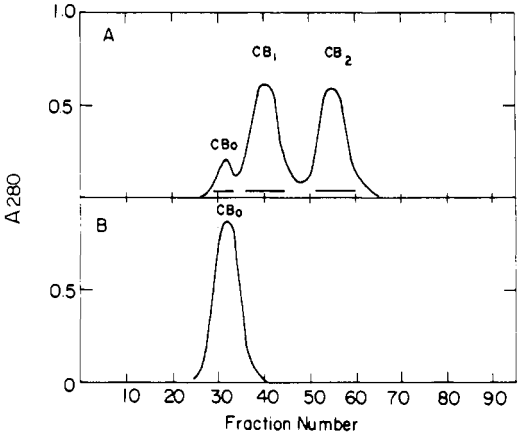

FIGURE 6: Fractionation of 8CM-α-lactalbumin derivative after treatment with CNBr. The protein (10 mg) was dissolved in 70% formic acid and treated with cyanogen bromide (200 equiv) for 24 hr at room temperature. The lyophilized product was dissolved in 0.1 *M* acetic acid, loaded onto a Sephadex G-100 column, and eluted with 0.1 *M* acetic acid at a rate of 50 ml/hr, 4 ml/fraction. (A) 8CM-α-lactalbumin; (B) oxidized 8CM-α-lactalbumin (see text).

Table I: Amino Acid Sequence Analysis of Acetyl-α-chymotrypsin Derivatives[a] after Cleavage with Cyanogen Bromide.[b]

Sequence Cycle No.	Amino Acid[c] Acetyl-α-chymotrypsin	Oxidized Acetyl-α-chymotrypsin[d]
1	Gly, Ile[e]	Ile
2	Asp	
3	Ser, Ala	Ala
4	Gly	Gly
5	Gly, Ala	Ala

[a] α-Chymotrypsin derivative (30 mg) was acetylated with 2000-fold excess acetic anhydride at pH 7–8 for 1 hr at room temperature. [b] Acetyl-α-chymotrypsin derivative (10 mg) was cleaved with CNBr in 70% formic acid for 24 hr at room temperature. [c] Determined as Pth-amino acids after automatic sequence analysis (see Methods). [d] This derivative contained one methionine and one methionine sulfoxide residue per protein molecule. [e] The amino acid sequences following methionine residues in α-chymotrypsin are: Met[180]-Ile-Cys-Ala-Gly-Ala; Met[192]-Gly-Asp-Ser-Gly-Gly (Brown and Hartley, 1966).

polypeptides may be quantitatively oxidized with NCS and chloramine-T. We then sought to determine whether these reagents can serve as probes for exposed methionine residues in native proteins.

When α-chymotrypsin was oxidized with 3 equiv of NCS at pH 8.5 one of its two methionine residues was oxidized (Figure 7A). Additional 100 equiv of NCS could oxidize only about 10–20% of the second methionine residue of the enzyme and no other amino acid was modified. In order to identify the oxidized residue, the following experiment was carried out: α-chymotrypsin was first oxidized with 3 equiv of NCS at pH 8.5, only one of its methionine residues being oxidized to methionine sulfoxide. The oxidized protein was then acetylated with acetic anhydride (2000-fold excess reagent, pH 7–8 at room temperature for 1 hr); the amino-terminal residues of this enzyme (Cys[1], Ile[16], Ala[149]) were acetylated, as verified by automatic sequence analysis. (No amino terminals were found). The oxidized acetylated protein was cleaved with cyanogen bromide (200-fold excess reagent in 70% formic acid at room temperature for 24 hr) and the resulting peptide mixture subjected to automatic sequence analysis. The results are presented in Table I, wherein it may be seen that both methionyl peptide bonds in acetylated α-chymotrypsin were cleaved with cyanogen bromide. Cleavage yields (estimated by quantitative determination of Pth-Ile and Pth-Gly by gas–liquid chromatography) were high; the Met–Ile bond was cleaved in 95% yield and the Met–Gly bond was cleaved in 92% yield. However, in NCS-oxidized α-chymotrypsin only Met[180]–Ile peptide bond was cleaved with cyanogen bromide (in 94% yield), indicating that methionine-180 in α-chymotrypsin was *not* oxidized with NCS. The Met[192]–Gly peptide bond in oxidized α-chymotrypsin was completely resistant to cleavage with cyanogen bromide, indicating that Met[192] had been oxidized and therefore did not react with cyanogen bromide.

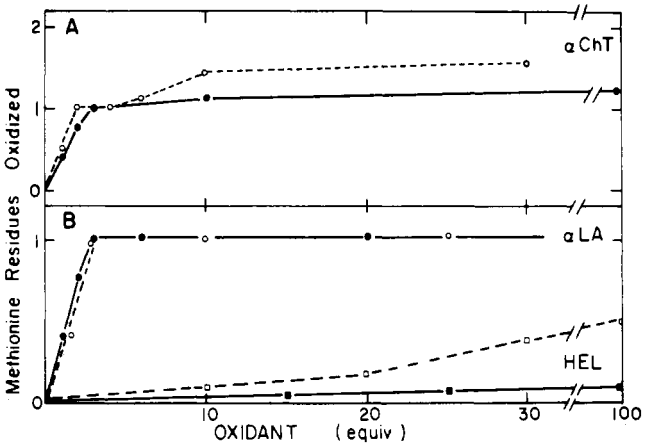

FIGURE 7: Oxidation of methionine residues in native proteins with increasing quantities of oxidants. The protein (2–3 mg) was dissolved in 1.0 ml of 0.1 *M* Tris-HCl (pH 8.5) and an aqueous solution of the oxidant (10 μmol) was added to it. After 20 min the protein was purified by gel filtration and analyzed for its Met and MetO content as described under Methods. (A) α-Chymotrypsin oxidized with NCS (●) and chloramine-T (○). (B) α-Lactalbumin (αLA) oxidized with NCS (●) and chloramine-T (○); lysozyme (HEL) oxidized with NCS (■) and chloramine-T (□).

With chloramine-T at pH 7.0–8.5 the results were somewhat different. With 2–6 equiv of chloramine-T, one methionine residue was again oxidized (Met[192]), but the second methionine residue could also be partially oxidized when larger quantities of chloramine-T (100 equiv) were used (Figure 7A).

Oxidation of Several Other Proteins with NCS and Chloramine-T. Several proteins were subjected to oxidation with NCS and chlormine-T at pH 8.5. As shown in Figure 7B, methionine residues of hen egg-white lysozyme were completely protected from oxidation by NCS at pH 8.5. With chloramine-T, under identical conditions, some oxidation of methionine was observed (less than 0.4 residue with 30 equiv of chloramine-T). The single methionine residue of α-lactalbumin from bovine milk was also exposed and could be oxidized with 3 equiv of NCS or chloramine-T (Figure 7B).

One of the four methionine residues of bovine pancreatic ribonuclease was sensitive to oxidation with NCS and chloramine-T although 15 equiv of these oxidants had to be used in order to oxidize this residue at pH 8.5 within 15 min (Figure 5B).

Discussion

The present study indicates that among the amino acids commonly present in non-SH proteins, methionine is the

residue most sensitive to oxidation with *N*-bromosuccinimide, *N*-chlorosuccinimide, or chloramine-T. Similar results were obtained with other oxidants, such as hydrogen peroxide (Neumann, 1972), 2,4,6-tribromocyclohexadienone (Burstein and Patchornik, 1972), and BNPS-skatole (Omenn et al., 1970). It has been demonstrated that with NBS, NCS, or chloramine-T rates of oxidation of methionine were pH-independent, whereas those of tryptophan, tyrosine, histidine, and cystine were pH-dependent.

NCS was found to have milder oxidation properties than NBS. Under acidic and neutral conditions, NBS would rapidly modify methionine, tryptophan, tyrosine, histidine, cysteine, and cystine residues, whereas NCS (under identical conditions) would oxidize methionine, tryptophan, and cysteine residues. Tyrosine, histidine, and cystine were not modified. Chloramine-T under acidic conditions oxidized cysteine, methionine, and tryptophan. However, at neutral and slightly alkaline pH (7.0–8.5), only cysteine and methionine residues were oxidized to cystine and methionine sulfoxide, respectively. Acting on free amino acids, polypeptides, or denatured proteins (at neutral pH), 1–3 equiv of NCS or chloramine-T were needed for quantitative oxidation of a methionine residue to methionine sulfoxide within 10 min. The results differed with native proteins. In α-lactalbumin, the single methionine residue was oxidized with 3 equiv of NCS or chloramine-T, indicating that this residue was completely exposed and therefore available for oxidation. On the other hand, hen egg-white lysozyme was completely resistant to oxidation with 100 equiv of NCS, indicating that the methionine residues of the protein were buried within the interior of the protein molecule, and were therefore unavailable for oxidation.

With α-chymotrypsin, it has been shown (Koshland et al., 1962; Schachter and Dixon, 1964; Weiner et al., 1966) that under *acidic* conditions Met^{192} could be oxidized with hydrogen peroxide while Met^{180} resisted such oxidation. With NCS, at pH 8.5, we observed a similar phenomenon; 3 equiv of NCS were sufficient to oxidize Met^{192} to methionine sulfoxide, while even 100 equiv of NCS could not oxidize Met^{180}.

In reduced and *S*-carboxymethylated ribonuclease, all four methionine residues were available for oxidation with NCS or chloramine-T. In the native enzyme, only one methionine residue could be oxidized, requiring 15 equiv of NCS or chloramine-T.

From the above examples, it appears that NCS at pH 7.0–8.5 is capable of "distinguishing" between three main classes of methionine residues in proteins: *exposed methionines* are those residues which can be oxidized with 1–3 equiv of NCS per residue; *buried* methionine residues resist oxidation with up to 100 equiv of NCS per residue; and *partially exposed* methionine residues can be oxidized with 10–20 equiv. As for the selectivity of this reagent, we have shown that under neutral and slightly alkaline conditions, with free amino acids and polypeptides and using large excesses of NCS, tryptophans were also oxidized. Although we did not detect any oxidation of tryptophans in native α-chymotrypsin or α-lactalbumin (using 10–30 equiv of NCS), such oxidation, however, should take place in proteins having "exposed" or "superactive" tryptophan residues.

Chloramine-T is more selective than NCS. Under neutral and slightly alkaline conditions it does not modify tryptophans while methionine residues are oxidized to methionine sulfoxides. However, due to its aromatic moiety it is capable (when used in high concentrations) of penetrating into hydrophobic regions of a protein, causing oxidation of buried methionine residues as well. High concentrations of chloramine-T (over 30 equiv) caused partial oxidation of the second methionine residue of α-chymotrypsin and an extra methionine residue in ribonuclease. Most existing methods for chemical modification of methionine residues in proteins take place in acidic pH, and therefore are not always applicable for distinguishing exposed methionine residues in proteins which undergo conformational changes or irreversible denaturation in acidic media. This modification of methionine residues, described above, is performed at neutral pH and therefore has a great potential for studying the role of this amino acid in the biological activity of proteins, as well as identifying exposed and buried methionine residues in proteins under physiological conditions.

Another potential use for this oxidation reaction is in the production of methionyl overlapping peptides for automatic amino acid sequence analyses. We have demonstrated with α-chymotrypsin that Met^{192} was oxidized and therefore did not cleave with cyanogen bromide. Met^{180} was resistant to oxidation and was therefore cleaved with cyanogen bromide. By automatic sequence analysis of oxidized and cyanogen bromide cleaved α-chymotrypsin we analyzed residues Ile^{181} through Met^{192}-Gly^{193} and obtained the desired sequence of the overlapping methionyl peptide.

In this study, we have utilized a novel method for quantitative determination and location of methionine sulfoxide residues in the presence of methionyl residues in proteins (Y. Shechter and Y. Burstein, to be published). In this procedure the oxidized protein is treated with CNBr, methionine residues are cleaved and converted to homoserines, while the methionine sulfoxides remain intact. During acid hydrolysis of the oxidized and cyanogen bromide-treated protein, methionine sulfoxides are reduced back and determined as methionines while the amount of homoserine (and its lactone) corresponds to that of the unoxidized methionine in the original sample.

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Bovine Brain Adenosine 3′,5′-Monophosphate Dependent Protein Kinase. Mechanism of Regulatory Subunit Inhibition of the Catalytic Subunit†

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ABSTRACT: Adenosine 3′,5′-monophosphate (cAMP) dependent protein kinase (EC 2.7.1.37) catalyzes the phosphorylation of serine and threonine residues of a number of proteins according to the following chemical equation: ATP + protein → phosphoprotein + ADP. The DEAE-cellulose peak II holoenzyme from bovine brain, which is composed of regulatory and catalytic subunits, is resistant to ethoxyformic anhydride inactivation. After adding cAMP, the protein kinase becomes susceptible to ethoxyformic anhydride inhibition. Ethoxyformic anhydride (2 m*M*) inhibits the enzyme 50% (5 min, pH 6.5, 30°) in the presence of 10 μM cAMP, but less than 5% in its absence. The substrate, Mg^{2+}-ATP, protects against inactivation suggesting that inhibition is associated with modification of the active site. Addition of regulatory subunit or Mg^{2+}-ATP to the isolated catalytic subunit also prevents ethoxyformic anhydride inactivation. These results suggest that the regulatory subunit shields the active site of the catalytic subunit thereby inhibiting it. In contrast to the bovine brain or muscle DEAE-cellulose peak II holoenzyme, the bovine muscle peak I holoenzyme is susceptible to ethoxyformic anhydride inactivation in the absence of cAMP.

Protein kinase (EC 2.7.1.37) catalyzes the phosphorylation of polypeptidic serine and threonine hydroxyl groups according to the chemical equation: ATP + protein → phosphoprotein + ADP. Through phosphorylation, protein kinases regulate the activity of many enzymes including phosphorylase kinase (Walsh et al., 1968), glycogen synthetase (Soderling et al., 1970), and hormone-sensitive lipase (Huttunen et al., 1970). They also appear to be involved in membrane permeability (Johnson et al., 1972), chromosome function (Langan, 1968), protein synthesis (Eil and Wool, 1973), and steroidogenesis (Walton et al., 1971).

A major class of protein kinase requires adenosine 3′,5′-cyclic monophosphate (cAMP)[1] for activity. The mechanism of activation is expressed by the equation RC +

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[1] Abbreviations used are: cAMP, adenosine 3′,5′-monophosphate; Mops, morpholinopropanesulfonic acid.