The Basic Trypsin Inhibitor of Bovine Pancreas. II. Alteration of the Methionine Residue*

BEATRICE KASSELL

From the Biochemical Laboratory for Cancer Research, Marquette University School of Medicine, Milwaukee, Wisconsin, 53233

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The single methionine residue of the basic pancreatic trypsin inhibitor is resistant to alkylation by iodoacetic acid at *p*H 2.7, probably indicating steric hindrance. However, hydrogen peroxide readily oxidizes this methionine to the sulfoxide. The protein containing the methionine sulfoxide group without other significant changes in the molecule is easily separated from the products of further reaction by chromatography on Amberlite IRC-50 (XE-64); the position of the protein peak is the same as for native inhibitor. The specific activity of the inhibitor does not decrease during peroxide oxidation, but on rechromatography the reaction product fails to achieve as high activity as native inhibitor.

The presence of a single methionine residue in the pancreatic trypsin inhibitor (Kassell *et al.*, 1963) stimulated interest in its possible role in the activity of the inhibitor. The hope of specific chemical alteration of this methionine residue was enhanced by the absence of histidine and tryptophan in this protein.

Neumann *et al.* (1962) developed two methods of chemical alteration and subsequent analysis of the methionine residues of ribonuclease, namely, reaction with iodoacetic acid to give carboxymethylsulfonium derivatives and oxidation with hydrogen peroxide to give methionine sulfoxide residues.

This paper deals with the application of these reactions to the pancreatic inhibitor.

EXPERIMENTAL

The preparation of inhibitor was the same as that described previously (Kassell *et al.*, 1963). Amino acids were determined with a Spinco amino acid analyzer, after hydrolysis of the samples either with 5.7 N HCl at 110° in sealed evacuated tubes for 24 hours, or with sodium hydroxide as described by Neumann *et al.* (1962). Activity of the inhibitor was determined by the Schwert and Takenaka (1955) method, except that 0.01 M $CaCl_2$ was added to the buffer (Kassell *et al.*, 1963).

Reaction with Iodoacetate

Procedure.—The method of Neumann *et al.* (1962) was followed. Solutions of inhibitor (30 mg) and iodoacetic acid (30 mg), each in 5 ml of water, were adjusted separately to *p*H 2.7 with formic acid, prewarmed to 40°, and mixed. Samples were removed at intervals for assay during 3 hours' incubation at 40°. The reaction was terminated by passage of the solution through a 1 × 5-cm column of Amberlite IRC-50 (XE-64) equilibrated with 5% acetic acid. The column was washed with 5% acetic acid until a baseline reading of A_{280} was reached, to remove excess reagent and salt, and the protein was then eluted with 50% acetic acid (Dixon, 1959). The acetic acid was removed from the pooled protein by dialysis in acetylated tubing followed by lyophilization (Kassell *et al.*, 1963). A portion of the lyophilized material was purified by chromatography on a 0.7 × 20-cm column of Amberlite IRC-50 (XE-64) in 0.2 M phosphate buffer of *p*H 6.83, desalted, and dialyzed, all in the same manner as in the purification of the original inhibitor.

The extent of reaction with iodoacetic acid was determined by amino acid analysis after performic acid oxidation of the protein samples (Neumann *et al.*, 1962). During this step methionine which has not reacted with iodoacetic acid is oxidized to the sulfone, but the carboxymethylsulfonium derivative resulting from reaction with iodoacetic acid resists oxidation. During acid hydrolysis (Gundlach *et al.*, 1959) the carboxymethylsulfonium derivative is reconverted to methionine, whereas the sulfone remains.

Results.—Treatment with iodoacetic acid caused no significant change in specific activity: the amount of trypsin inhibited by 1 μg of inhibitor was 2.29 μg at zero time and 2.34 μg at 3 hours. In Figure 1 chromatography of the reaction mixture (solid line) is shown in comparison to untreated inhibitor (dotted line). Amino acid analysis (Table I) for the total reaction mixture did not differ significantly from that for peak 2 of Figure 1. About 90% of the methionine (determined as methionine sulfone) was unaffected by the iodoacetate treatment. The remaining 10% (determined in the hydrolysate as methionine) was converted to the carboxymethylsulfonium derivative. This admixture of about 10% of altered protein is reflected in the width of peak 2 in Figure 1 compared to the peak from control inhibitor, particularly the more pronounced tailing. By comparison to the chromatogram of Neumann *et al.* (1962, Fig. 1) for ribonuclease derivatives it is likely that the altered inhibitor was responsible for the tail portion of the peak. The activity determinations are not sufficiently accurate to state whether the 10% of altered inhibitor is active or not.

The determination of half-cystine plus cysteic acid after oxidation indicates the loss of about one-half a residue in comparison with the control: 5.29 and 5.20 residues as compared to 5.87 residues. The amount of material in peak 1 of Figure 1 was too small for a separate analysis. From experience with impure inhibitor, and from the results with peroxide oxidation given below, it is probable that peak 1 contained inhibitor in which a part of the cystine and tyrosine had been altered by traces of iodine in the iodoacetic acid.

Oxidation with Hydrogen Peroxide

Procedure.—Two sets of conditions were used: (a) similar to those of Neumann *et al.* (1962), and (b) more severe ones, in order to identify the products of side reactions.

(a) A solution of inhibitor, 10 mg/ml, adjusted to

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TABLE I

THE EFFECT OF IODOACETIC ACID ON THE AMINO ACID COMPOSITION OF THE INHIBITOR

	Number of Amino Acid Residues per Molecule[a]			
Constituent in Protein →	Carboxymethyl-sulfonium Derivative of Methionine	Unchanged Methionine	Cystine	
Determined as →	Methionine	Methionine Sulfone	Cys/2	Cysteic Acid
Oxidized inhibitor (control)	Trace	0.88	0.39	5.48
Oxidized reaction mixture	0.07	0.69	0.93	4.36
Oxidized peak 2 of Fig. 1	0.09	0.68	0.75	4.55

[a] The *untreated* protein contains 6 residues of half-cystine and 1 of methionine. It has a molecular weight of 6513. Total amino acid analysis of the *treated* samples showed a very slight loss of arginine and lysine (about 0.3 residue each) and a small increase in ammonia in the hydrolysate. There was loss of tyrosine, but not a larger loss than in the control, indicating that the loss was due to the performic acid oxidation step. Other amino acids were not affected.

TABLE II

SPECIFIC ACTIVITY[a] OF THE INHIBITOR DURING TREATMENT WITH H_2O_2

	Micrograms Trypsin Inhibited per Microgram Inhibitor			
	Experiment (a)[b] (*p*H 2.1, 30°, 0.3 M H_2O_2)		Experiment (b)[b] (*p*H 1.0, 50°, 1 M H_2O_2)	
Minutes	Control[c]	Treated	Control[c]	Treated
0	2.52	2.30	1.91	2.17
12		2.48		2.17
30	2.60	2.61	2.08	2.17
60	2.59	2.34	2.03	1.82

[a] The error is estimated to ±10% for single determinations. [b] (a) and (b) refer to procedure used (under Experimental). [c] Kept under the same conditions without H_2O_2.

TABLE III

THE EFFECT OF H_2O_2 ON METHIONINE AND TYROSINE [ALKALINE HYDROLYSIS]

Experiment	Protein	Methionine	Methionine Sulfoxide	Tyrosine
a	Treated	None	0.84	3.2
b		0.02	0.84[a]	3.1
a	Control	0.70	0.19[b]	3.3
b		0.65	0.24[b]	3.3

[a] Plus a trace of methionine sulfone, absent in the other samples. [b] The high methionine sulfoxide content of the controls may be due to hydrolysis in capped Teflon tubes (Neumann *et al.*, 1962) without total exclusion of air, or to the sensitivity of the methionine of the inhibitor to the long preparative procedure.

*p*H 2.1 with perchloric acid, and 0.3 M with respect to hydrogen peroxide, was incubated at 30° for 70 minutes. Samples were removed periodically for activity assay. The reaction was stopped by adjustment to *p*H 7 with 1 N NaOH (buffering with 0.02 M final concentration of *p*H 7.0 sodium phosphate buffer) and addition of 5-μl portions of 1% catalase solution (Worthington Biochemical Corp., Freehold, N. J.) at 2-minute intervals until bubbling stopped. The solution was dialyzed and lyophilized, and a portion was chromatographed (Fig. 2) on IRC-50 as described above for the products of reaction with iodoacetic acid.

(b) The procedure was the same as (a) except that the solution was incubated at *p*H 1.0 and 50° in 1 M hydrogen peroxide (chromatography, solid line, Fig. 3).

Amino acid analysis was performed on the product

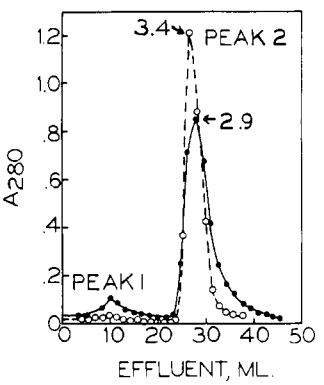

FIG. 1.—Chromatography of the products of reaction of the inhibitor with iodoacetate. Solid line, reaction products: column, 0.7 × 21 cm of IRC-50, (XE-64); eluent, 0.2 M phosphate buffer of *p*H 6.83; load 8.5 mg.; fractions 1.6 ml; rate about 10 ml/hr. Dotted line, untreated inhibitor; load 6.4 mg under the same conditions. The figures on the curves are activities determined within 1 hour after chromatography and expressed as μg trypsin inhibited per μg of inhibitor.

from experiment (a) before chromatography. For experiment (b) the separate peaks of Figure 3 were used. Methionine sulfoxide is unstable during acid hydrolysis, and mainly reverts to methionine; it is determined after alkaline hydrolysis (Neumann *et al.*, 1962). The other amino acids were determined in acid hydrolysates.

Results.—Chromatography of the reaction products of experiment (a) (Fig. 2) separated an impurity in a preceding peak which was too small to analyze. Under the more severe conditions (b), sufficient material (Fig. 3) was obtained for separate analysis of each peak. The position of the methionine sulfoxide inhibitor did not differ significantly from that of the native protein shown by the dotted line, being in one case about 1 ml ahead, and in the other about 1 ml behind.

The specific activity of the native inhibitor decreases on standing in phosphate buffer or on lyophilization, but the activity is restored to the maximum values so far found (3.3–3.7 μg of trypsin inhibited/μg of inhibitor) by rechromatography under the same conditions as used here (Kassell *et al.*, 1963). The mechanism of this loss and restoration is unknown.

The preparation of inhibitor used for the peroxide oxidation had already been lyophilized, and had a rather low specific activity (Table II). This activity did not change significantly during reaction with H_2O_2 (Table II), with the possible exception of the 60-minute analysis in experiment (b). However there was a noticeable difference in the restoration of activity

TABLE IV
THE EFFECT OF H_2O_2 ON AMINO ACID COMPOSITION [ACID HYDROLYSIS]

Experiment	Protein	Number of Amino Acid Residues per Molecule[a]				
		Methionine	Methionine Sulfoxide	Cys/2	Cysteic Acid	Tyrosine
a	Treated[b]	0.84[c]	Trace	5.11	0.26	3.4
Reaction mixture	Control	0.88	None	6.02	None	3.8
b						
Separate peaks	Peak 1[d]	0.07	0.59[e]	None	3.3	1.8
of Fig. 3	Peak 2[b]	0.79[c]	None	5.39	0.07	3.4

[a] The *untreated* protein contains 6 residues of half-cystine, 1 of methionine, and 4 of tyrosine. It has a molecular weight of 6513. [b] Other amino acids were not affected. [c] Methionine sulfoxide reverted to methionine during acid hydrolysis. [d] Small amounts of aspartic acid, phenylalanine, and proline were lost in addition. [e] Plus a trace of methionine sulfone.

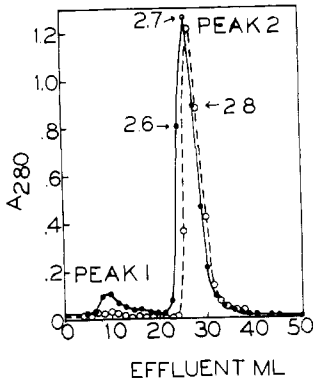

FIG. 2.—Chromatography of the products of reaction of the inhibitor with hydrogen peroxide under mild conditions. Solid line: see text, under Experimental (a); load 10.0 mg; other conditions as in Fig. 1. Dotted line: same experiment as in Fig. 1.

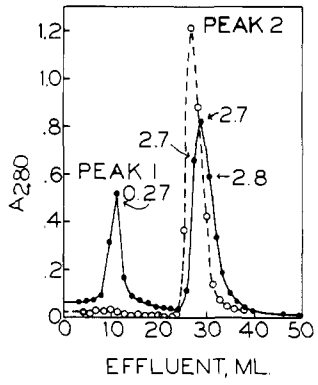

FIG. 3.—Chromatography of the products of reaction of the inhibitor with hydrogen peroxide under severe conditions. Solid line: see text, under Experimental (b), load 10.0 mg; other conditions as in Fig. 1. Dotted line: same experiment as in Fig. 1.

after chromatography. The chromatographed, peroxide-treated inhibitor gave at most 80% of the activity of the corresponding native inhibitor (Figs. 2 and 3).

Amino acid analysis after alkaline hydrolysis (Table III) showed that in both experiments the methionine had been almost quantitatively converted to the sulfoxide. During acid hydrolysis (Table IV) of the reaction products of experiment (a) practically all of the methionine sulfoxide was reconverted to methionine, as expected. The same was true for peak 2 of experiment (b). However, with the protein of peak 1, most of the methionine sulfoxide remained in the hydrolysate, and was determined as such.

Side reactions which occurred under the milder conditions (a) were some oxidation of cystine and slight loss of tyrosine (Table IV). Under the more severe conditions (b) in the protein of peak 1 (Fig. 3), the cystine had disappeared completely, with only a little more than half recovered as cysteic acid. More than half the tyrosine was also missing, and small amounts of aspartic acid, phenylalanine, and proline were lost. The analysis for peak 2 of experiment (b) was very similar to the material obtained from the milder experiment, and showed little destruction of either cystine or tyrosine.

DISCUSSION

The difference in the susceptibility of the methionine residue to the two reagents is marked: with iodoacetic acid only 0.1 residue was converted to the carboxymethylsulfonium derivative, while with hydrogen peroxide there was practically quantitative conversion to the sulfoxide. This probably indicates that this residue is inaccessible, even at *p*H 2.7, to a reagent the size of iodoacetic acid. Neumann *et al.* (1962) found the alkylation reaction to occur only when ribonuclease was denatured; perhaps the inhibitor is not unfolded at this *p*H. It is of interest that the methionine residues of myoglobin were found by X-ray analysis to be internal (Kendrew *et al.*, 1961).

Side reactions under mild conditions of peroxide oxidation were minimal, and may be even less than they appeared. The loss of cystine and tyrosine shown in Tables III and IV may not all have been due to side reactions during the oxidation. During acid hydrolysis of proteins in the presence of methionine sulfoxide there is some oxidation of cystine and tyrosine (Neumann *et al.*, 1962). Most of the loss of cystine may therefore have occurred during hydrolysis (total loss was less than 1 half-cystine). Since for tyrosine, however, the results of acid and alkaline hydrolysis are in good agreement, it is likely that the change was already present before hydrolysis.[1] The protein of peak 1 of Figure 3, which contained no cystine, yielded most of the methionine sulfoxide unchanged after acid hydrolysis, directly confirming the interaction of these two compounds (Neumann *et al.*, 1962).

There was no loss of activity during the mild peroxide oxidation. That the methionine residue is not essential for activity in this trypsin inhibitor is not surprising, in view of the absence of methionine in the lima bean trypsin inhibitors (Jones *et al.*, 1963). However, the methionine residue plays some role in the activity, since the restoration of activity after rechromatography of the methionine sulfoxide–con-

[1] Tyrosine is known to be oxidized by H_2O_2 in HCl solution, probably to 3-chlorotyrosine (Kirby, 1962). Although an effort was made to avoid chlorine contamination, there was apparently enough present to give some reaction due to the chlorine formed in the presence of H_2O_2.

taining inhibitor reached a level only about 80% of that reached with native inhibitor.

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Conformational Studies on Synthetic Poly-α-amino Acids: Factors Influencing the Stability of the Helical Conformation of Poly-L-glutamic Acid and Copolymers of L-Glutamic Acid and L-Leucine*

GERALD D. FASMAN,† CAROLE LINDBLOW, AND ERIKA BODENHEIMER

From the Graduate Department of Biochemistry,‡ Brandeis University, Waltham 54, Massachusetts

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Optical rotatory dispersion studies were performed on poly-L-glutamic acid and copolymers of L-glutamic acid and L-leucine as a function of temperature in order to measure the relative stabilities of their helical conformations toward a temperature induced helix → random-coil transition. The copolymers were found to have the greater stability. An inverse temperature effect is reported, wherein the helical content of several of these copolymers first decreased and then increased with increasing temperature. The temperature of inversion is dependent on the leucine composition and at a leucine content of 33% the copolymer shows only an increase in helicity upon heating. The behavior of these copolymers could best be explained in terms of hydrophobic interactions. The effect of ionic strength, urea, LiBr, sodium dodecylsulfate, dioxane, and ethylene glycol on the helical content of these protein models is reported. LiBr (1 M) completely destroyed the poly-L-glutamic acid helix while 8 M urea had little effect. The copolymers were more resistant to the effects of LiBr and slightly less resistant toward urea than was the homopolymer. Sodium dodecylsulfate produced minor effects on the polymers. Helical stabilization through carboxyl-carboxyl interactions was implicated. The addition of nonaqueous solvents (dioxane, ethylene glycol, chloroethanol) to both model systems in water caused increased $-b_0$ values and produced a stabilization toward the temperature-induced transition. As extremely high $-b_0$ values were obtained (-700 for poly-L-glutamic acid, -900 for a copolymer) in these organic-aqueous solutions, the justification of estimating helical content in such mixtures, based on optical rotatory measurements, is open to question.

The analogy between the physical-chemical properties of synthetic poly-α-amino acids and proteins has provided ideal research models for the investigation of the factors responsible for conformational stability of proteins (for reviews see Katchalski and Sela, 1958; Urnes and Doty, 1961). The first optical rotatory dispersion studies on synthetic polypeptides by Moffitt and Yang (1956) were carried out on poly-γ-benzyl-L-glutamate and poly-L-glutamic acid, and these studies established the basis upon which much fruitful work has since been done. The synthesis of high-molecular-weight water-soluble poly-L-glutamic acid (Blout and Idelson, 1956; Idelson and Blout, 1958) enabled the study of the helix → random-coil transition in several solvent systems (Doty *et al.*, 1957; Blout and Idelson, 1956; Idelson and Blout, 1958; Goldstein and Katchalski, 1960; and Wada, 1960). As poly-L-glutamic acid was the model polypeptide upon which the early work on optical rotatory dispersion was done, it was decided to use this polymer as the standard for examining the relative stability of the α-helix under varying conditions and to compare this work with a study of the effect of the incorporation of an amino acid with a branched hydrocarbon side chain, leucine, on helical stability. A preliminary report of these studies has been published (Fasman *et al.*, 1962). This study was undertaken because of the importance placed in recent years on the role that hydrophobic forces play in stabilization of native structures (Kauzmann, 1959; Scheraga, 1960, 1961; Klotz, 1960; Scheraga *et al.*, 1962; Nemethy and Scheraga, 1962a,b,c; Fasman, 1962; Nemethy *et al.*, 1963; Tanford *et al.*, 1960; Tanford and De, 1961; Tanford, 1962a; Foss, 1961; Warner, 1961; Lowey and Cohen, 1962). The contribution of the side-chain interactions in myoglobin has recently been shown to be of importance in the native structure (Kendrew, 1962). The consequence of the interaction of nonpolar side chains with each other and with the aqueous solvent have been examined thermodynamically (Kauzmann, 1959; Scheraga, 1960, 1961; Klotz,

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