Biomedical Press, Amsterdam.
Elzinga, M., Collins, J. H., Kuehl, W. M., & Adelstein, R. S. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2687–2691.
Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.
Fine, R. E., & Bray, D. (1971) *Nature (London), New Biol. 234*, 115–118.
Garrels, J. I., & Gibson, W. (1976) *Cell 9*, 793–805.
Gerday, C., Robyns, E., & Gosselin-Rey, C. (1968) *J. Chromatogr. 38*, 404–411.
Gordon, D. J., Eisenberg, E., & Korn, E. D. (1976) *J. Biol. Chem. 251*, 4778–4786.
Gordon, D. J., Boyer, J. L., & Korn, E. D. (1977) *J. Biol. Chem. 252*, 8300–8309.
Gruenstein, E., & Rich, A. (1975) *Biochem. Biophys. Res. Commun. 64*, 472–477.
Higashi, S., & Oosawa, F. (1965) *J. Mol. Biol. 12*, 843–865.
Kane, R. E. (1975) *J. Cell Biol. 66*, 305–315.
Kasai, M. (1969) *Biochim. Biophys. Acta 180*, 399–409.
Laitinen, H. A. (1960) *Chemical Analysis, An Advanced Text and Reference*, pp 546–548, McGraw-Hill, New York.
Lee, J. C., Frigon, R. P., & Timasheff, S. N. (1973) *J. Biol. Chem. 248*, 7253–7262.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Moring, S., Ruscha, M., Cooke, P., & Samson, F. (1975) *J. Neurobiol. 6*, 245–255.
Murphy, A. J. (1971) *Biochemistry 10*, 3723–3728.
Nagy, B., & Strzelecka-Golaszewska, H. (1972) *Arch. Biochem. Biophys. 150*, 428–435.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.
Olmsted, J. B., & Borisy, G. G. (1975) *Biochemistry 14*, 2996–3005.
Pardee, J. D. (1978) Ph.D. Thesis, Colorado State University, Fort Collins, CO.
Pardee, J. D., & Bamburg, J. R. (1976) *J. Neurochem. 26*, 1093–1098.
Puszkin, S., & Berl, S. (1972) *Biochim. Biophys. Acta 256*, 695–709.
Puszkin, S., Berl, S., Puszkin, E., & Clarke, D. D. (1968) *Science 168*, 170–171.
Rubenstein, P. A., & Spudich, J. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 120–123.
Spudich, J. A. (1974) *J. Biol. Chem. 249*, 6013–6020.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.
Storti, R. V., & Rich, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2346–2350.
Vandekerckhove, J., & Weber, K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1106–1110.
Whalen, R. G., Butler-Browne, G. S., & Gros, F. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2018–2022.
West, J. J., Nagy, B., & Gergely, J. (1967) *Biochem. Biophys. Res. Commun. 29*, 611–616.
Yang, Y., & Perdue, J. F. (1972) *J. Biol. Chem. 247*, 4503–4509.

Structure of Potato Carboxypeptidase Inhibitor: Disulfide Pairing and Exposure of Aromatic Residues†

T. Richard Leary, Donald T. Grahn, Hans Neurath,* and G. Michael Hass

ABSTRACT: The determination of the covalent structure of a carboxypeptidase inhibitor from potatoes containing 39 amino acid residues has been completed by analysis of the pairing of the six half-cystine residues. Since the native inhibitor is resistant to fragmentation by proteases, the protein was first subjected to cleavage at aspartic acid residues by exposure to 0.03 N HCl at 110 °C for 10 h to yield a fragment containing two chains (residues 6–15 and residues 18–39) held together by three disulfide bonds. Digestion with subtilisin and Pronase, respectively, yielded sets of peptides from which, by diagonal electrophoresis and amino acid analysis, the paired cystinyl residues were identified as Cys-8 to Cys-24, Cys-12 to Cys-27, and Cys-18 to Cys-34. Charge-transfer titration of the native inhibitor with *N*-methylnicotinamide chloride suggests that one of the two tryptophan residues and the single tyrosine residue are exposed to the solvent.

Protein inhibitors of proteases are widely distributed in plants and animals. Many of these have been isolated and characterized by chemical, physicochemical, and enzymatic methods (Fritz et al., 1974; Laskowski & Sealock, 1971).

A protein inhibitor of carboxypeptidases A and B from Russet Burbank potatoes was described by Ryan (1971) and its amino acid sequence was reported by Hass et al. (1975). This inhibitor consists of a single polypeptide chain containing 39 amino acid residues with a molecular weight of 4300 (Hass et al., 1975). The protein contains six half-cystine and no cysteine residues (Figure 1) (Ryan et al., 1974).

Hass et al. (1976) studied the interaction of native and chemically modified potato inhibitor with carboxypeptidase A and found that the inhibitor became inactivated when the terminal α-carboxyl group was blocked by attaching a leucine residue. Reactivation occurred when this leucine residue was removed. No other modification appeared to significantly modify the activity of the inhibitor. However, a complex of carboxypeptidase with acetylated inhibitor protected the acetylated tyrosine-37 from deacylation with hydroxylamine,

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195 (T.R.L., D.T.G., and H.N.), and the Department of Bacteriology and Biochemistry, University of Idaho, Moscow, Idaho 83843 (G.M.H.). *Received January 5, 1979.* This work has been supported by grants from the National Institutes of Health to the University of Washington (GM 15731) and to the University of Idaho (GM 22748). Published with the permission of the Director of the Idaho Agricultural Experiment Station as Research Paper No. 78517.

0006-2960/79/0418-2252$01.00/0

implying that the carboxyl-terminal region of the inhibitor was in contact with the enzyme.

In this communication, we complete the study of the covalent structure of the potato carboxypeptidase inhibitor by describing the pairing of the six half-cystine residues of the protein. We also report on the exposure of aromatic residues as determined by charge-transfer titrations of the native protease with *N*-methylnicotinamide chloride.

Materials and Methods

The carboxypeptidase inhibitor was prepared from Russet Burbank potatoes by the method of Ryan et al. (1974). Subtilisin BPN′ was purchased from Teikoku Chemical Industry, Osaka, Japan. Thermolysin and "Pronase" (a mixture of proteases elaborated by *Streptomyces griseus*) were purchased from Calbiochem. *Myxobacter* AL-1 Protease II, which cleaves peptide bonds on the amino side of lysine residues (Wingard et al., 1972), was a gift from Dr. Gene K. Zwolinski, Case Western Reserve University, Cleveland. Sephadex G-25SF and SP-Sephadex C-25 were used according to the manufacturer's specifications. Pyridine was distilled from ninhydrin prior to use in chromatographic and electrophoretic systems (Hill & Delaney, 1967). All other chemicals were reagent grade or better.

Amino Acid Analysis. Dried samples were suspended in constant-boiling HCl. The tubes were repeatedly evacuated and flushed with nitrogen before sealing, and the samples were hydrolyzed at 110 °C for 18–20 h. Analyses were performed on a Durrum amino acid analyzer (Model D-500). The recovery of cysteic acid in various pools and eluates was based on the cysteic acid content of the original oxidized samples and is reported as a percentage of the undegraded protein.

Paper Electrophoresis. High-voltage paper electrophoresis was performed on each peptide pool for 1.25 h at 2 kV using pyridine–acetate buffer, pH 3.5 (Bennett, 1967). For analytical purposes, peptides were detected by dipping the paper in 2 g of ninhydrin/L of acetone and warming of the chromatograms in an 80 °C humid oven. Tryptophan-containing peptides were identified by staining the electropherograms with *p*-dimethylaminobenzaldehyde (Smith & Birdenough, 1960). The diagonal electrophoretic technique of Brown & Hartley (1966) was used at pH 3.6 except that the second dimension was developed with 0.2 g of ninhydrin/L of acetone. Preparative samples were applied in amounts of approximately 100 nmol/cm.

Performic Acid Oxidation. The procedure outlined by Hirs (1967) was used, except that methanol was omitted and the oxidation was performed at 0 °C. Performic acid oxidation of the diagonal strips was performed as described by Brown & Hartley (1966).

Acidic Cleavage. Cleavage at aspartic acid residues was performed by hydrolysis in 0.03 N HCl at 110 °C for 10 h (Schultz, 1967).

Elution of Peptides from Paper. The peptide spots identified with diluted ninhydrin were cut out of the electropherogram, macerated, and placed in 1.2-mL polypropylene tubes of an Eppendorf microcentrifuge. Small holes were made in the tips of the tubes with a dissection needle. The polypropylene tubes were then mounted on 4-mL centrifuge tubes. The peptides were eluted from the shredded paper by applying successively 1.5 mL of 10%, 10%, and 20% (v/v) acetic acid and spinning the suspended particles in a table top clinical centrifuge for several minutes.

Sequenator analysis was carried out in a Spinco sequencer (Model 890A) by using the method of Edman & Begg (1967) as modified by Hermodson et al. (1972).

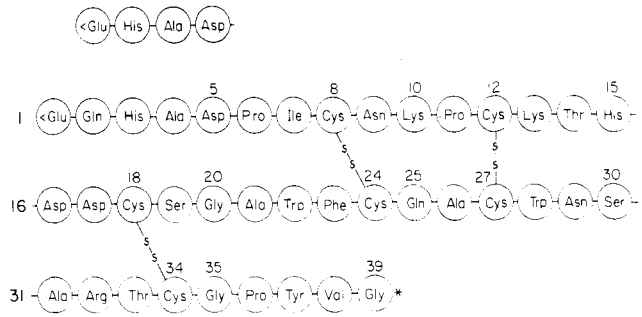

FIGURE 1: Amino acid sequence of potato carboxypeptidase inhibitor, showing the disulfide pairing of half-cystine residues. The tetrapeptide above residues 2–5 denotes the amino-terminal sequence of a minor compound of the inhibitor (Hass et al., 1975).

N-Methylnicotinamide Titration. The titrations were carried out at 21 °C by using the constant ionic strength procedure (1 M) of Hinman et al. (1974). All reagents were dissolved in 0.05 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes) buffer, pH 7.00. Four 1-cm pathlength matched cuvettes were used in place of tandem cells. Samples were scanned from 550 to 320 nm by using a Cary 15 spectrophotometer. The concentration of carboxypeptidase inhibitor (1.5 mM) was determined using the chloroacetyl-L-tyrosine assay described below.

Assay of Activity. The activity of the carboxypeptidase inhibitor was determined by assaying bovine carboxypeptidase A in the presence of predetermined amounts of inhibitor with chloroacetyl-L-tyrosine as a substrate (Hass et al., 1976).

Results

Preparation of a Fragment by Cleavage with Acid. Native inhibitor was resistant to fragmentation by proteases, but partial acid hydrolysis generated a fragment which was susceptible to proteolytic attack. Preliminary experiments indicated that incubation in 0.03 N HCl for 10 h at 110 °C released 3 equiv of aspartic acid and less than 0.2 equiv of any other free amino acid. For preparative purposes, the native inhibitor (50 mg) was suspended in 1 mL of 0.03 N HCl in a test tube which was then evacuated and sealed, and the suspension was incubated for 10 h at 110 °C. The contents were diluted with an equal volume of water and lyophilized. The lyophilized powder was dissolved in 4 mL of 9% (v/v) formic acid and fractionated on a column (1.5 × 95 cm) of Sephadex G-25SF equilibrated with 9% formic acid.

Sequenator analysis of the breakthrough fraction identified two major new amino-terminal sequences in essentially equal amounts, i.e., Pro-Ile-(Cys)[1]-Asn- (residues 6–9, Figure 1) and (Cys)[1]-Ser-Gly-Ala- (residues 18–21) and a minor sequence (6%) of Gly-Ala-Trp-Phe (residues 20–23). Since the native inhibitor is blocked at the amino terminus, these new major sequences must have arisen by acid-catalyzed cleavage of the Asp-Pro bond (residues 5–6) and the Asp-Cys bond (residues 17–18). The minor cleavage corresponds to partial hydrolysis of the Ser-Gly bond (residues 19–20). Amino acid analysis of the same breakthrough fraction revealed the loss of the following amino acids: $Glx_{1.4}$, Ala, His, Asp_3 (Table I). Since native inhibitor contains two species in equal concentration, one of which lacks a glutamine residue in position 2 (Figure 1), removal of residues 1–5 by partial acid hydrolysis would remove $Glx_{1.5}$, Ala, His, and Asp. The other two aspartyl residues which were lost during partial acid hydrolysis must

[1] Cys was not identified in the sequenator analysis and is placed by inference.

Table I: Comparison of Amino Acid Composition (Residues per Molecule)

	inhibitor[a]	acid-cleaved inhibitor
Lys	2	2.1
His	2	1.1
Arg	1	1.1
CM-Cys	6	ND
Asx	5	2.1
Thr	2	2.0
Ser	2	1.8
Glx	2.5	1.1
Pro	3	2.9
Gly	3	2.7
Ala	4	3.0
Val	1	0.9
Ile	1	1.0
Tyr	1	1.0
Phe	1	1.1
Trp	2	ND

[a] Ryan et al. (1974).

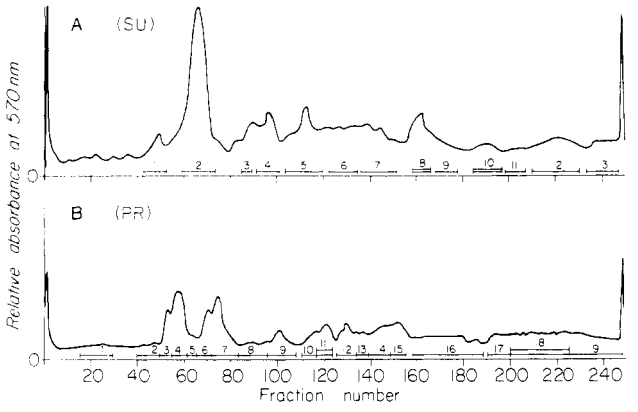

FIGURE 2: Elution profile of chromatography of subtilisin (SU) digest (A) and Pronase (PR) digest (B) of acid-treated carboxypeptidase inhibitor on SP-Sephadex C-25. The pooled fractions are indicated by the numbered horizontal bars. For experimental details, see the Results section.

be derived from residues 16 and 17.

The fragment prepared in this manner thus appears to consist primarily of two chains (residues 6–15 and residues 18–39). This fragment was the starting point for the following identification of disulfide bonds. The fragment was cleaned with either subtilisin or Pronase to generate smaller cystinyl peptides.

Cleavage with Subtilisin. The acid-cleaved inhibitor (4.8 μmol) was digested with 2.7 mg of subtilisin at pH 7.0 in a pH-stat at 37 °C for 2 h. The reaction mixture was lyophilized, dissolved in 0.05 M pyridine–acetate (pH 2.4), and applied to a column of SP-Sephadex C-25. The chromatogram (Figure 2) was developed with the gradient system described by Walsh et al. (1970).

Pooled fractions were tested for cystine by the method of Toennies & Kolb (1951). Fractions containing cystine were further examined by preparative diagonal electrophoresis as described by Brown & Hartley (1966).

By this procedure, fraction SU-8 (Figure 2) yielded two peptides, SU-8a and SU-8b. Elution and hydrolysis of these peptides (Table II) allowed unequivocal identification of Cys-34 and Cys-18 joined by a disulfide bond. Identical results were obtained by diagonal electrophoresis of fraction SU-10 (Table II).

The remaining cystine peptides were located in fraction SU-12. The amino acid composition was consistent with the presence of a single fragment containing residues 6–12 and

Table II: Peptides Derived by Diagonal Electrophoresis[a]

peptide	composition	% yield	identification of cystine
	Subtilisin Digest		
SU-8a	Arg (0.8), Thr (1.0), Cys (1.0)	14	Cys-34
SU-8b	Cys (0.9), Ser (1.1), Gly (1.2), Ala (1.0)	7	Cys-18
SU-10a	Arg (1.0), Thr (1.0), Cys (1.2)	25	Cys-34
SU-10b	Cys (1.2), Ser (0.9), Gly (1.0), Ala (1.1)	6	Cys-18
SUMT-1a	Ala (0.8), Cys (1.0)	19	Cys-27
SUMT-1b	Lys (0.7), Pro (0.9), Cys (1.0)	22	Cys-12
SUMT-2a	Cys (1.0), Glx (0.6)	4	Cys-24
SUMT-2b	Phe (1.0), Cys (1.0)	12	Cys-24
SUMT-2c	Ile (1.0), Cys (0.9), Asx (1.2)	20	Cys-8
	Phe (0.9), Cys (0.9), Glx (1.1)		Cys-24
SUMT-2d	Pro (0.9), Ile (1.0), Cys (1.3), Asx (1.1)	17	Cys-8
	Pronase Digest		
PR-11a	Cys (1.0), Ser (0.7), Gly (1.0)	21	Cys-18
PR-11b	Cys (1.0), Gly (1.0), Pro (0.9), Tyr (0.7)	19	Cys-34
PR-11c	Thr (0.7), Cys (0.9), Gly (1.0), Pro (1.0), Tyr (0.6)	4	Cys-34
PRT-18a	Ala (1.0), Cys (1.2)	11	Cys-27
PRT-18b	Lys (1.1), Pro (1.0), Cys (1.2)	8	Cys-12
PRA-1	Cys (2.0), Asx (1.2), Lys (1.8), Pro (1.0), Cys (1.0)	8	Cys-8, -12
PRA-2	Cys (1.6), Glx (0.9), Ala (1.0)	9	Cys-24, -27
PRA-3	Cys	2	--

[a] Data given as residues per molecule.

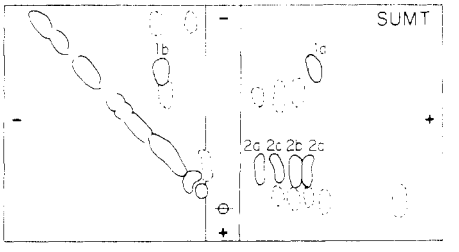

FIGURE 3: Two-dimensional electropherogram of peptides of pool SU-12. The unoxidized digest was first subjected to electrophoresis in the vertical dimension and then oxidized and electrophoresis was repeated in the horizontal dimension. For further details, see the Materials and Methods and Results sections.

23–27 connected by two disulfide bonds. This fragment was not susceptible to cleavage by either trypsin or chymotrypsin. To cleave and separate the two cystinyl peptides from each other, fraction SU-12 was treated with *Myxobacter* AL-1 Protease II which cleaves specifically on the amino side of lysyl residues and with thermolysin which was expected to cleave on the amino side of alanyl residues of peptide SU-12. Fraction SU-12 (500 nmol) was treated with 85 μg of the *Myxobacter* protease in 0.5 mL of 0.1 pyridine–acetate (pH 7) at 37 °C for 24 h, followed by incubation with 600 μg of thermolysin for 40 h. Subsequent diagonal electrophoresis of this reaction mixture yielded one pair of peptides, SUMT-1a and SUMT-1b (Figure 3), which contain Cys-27 and Cys-12, respectively (Table II). Thus, Cys-27 and Cys-12 are joined by a disulfide bond.

A second set of four peptides comigrated on the same electropherogram in the first dimension and separated after performic oxidation. These peptides (SUMT-2a through -2d) corresponded in composition to various cleavage products surrounding Cys-8 and Cys-24 (Table II). Cys-24 was found in SUMT-2a (Cys, Glx), SUMT-2b (Phe, Cys), and Cys-8 in SUMT-2d (Pro, Ile, Cys, Asx). However, the peptide SUMT-2c appeared to be a mixture of the two peptides (Ile,

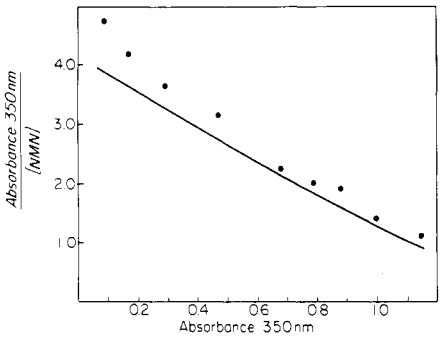

FIGURE 4: Titration of native carboxypeptidase with *N*-methylnicotinamide (NMN). For experimental details, see the text. The solid line is a theoretical titration curve, assuming that the extinction coefficient of *N*-methylnicotinamide–tryptophan complex is 880 M^{-1} cm^{-1}, that of the *N*-methylnicotinamide–tyrosine complex is 130, and that the protein concentration is 1.1 mM.

Cys, Asx) and (Phe, Cys, Glx), which have similar charge/mass ratios. These data are consistent with a disulfide bond between Cys-8 and Cys-24.

To confirm these identifications, the acid-cleaved inhibitor was separately digested with Pronase and a second set of cystinyl peptides examined.

Cleavage with Pronase. Acid-cleaved inhibitor (4 μmol) was dissolved in 10 mL of 2 mM $CaCl_2$ and digested with 1.25 mg of Pronase at pH 7.5 in a pH-stat at 37 °C for 30 min. The reaction mixture was adjusted to pH 2.4 with formic acid and fractionated on SP-Sephadex as described above. Fractions PR-11 and PR-18 contained cystine and yielded useful peptides.

Diagonal electrophoresis of fraction PR-11 yielded three peptides. Whereas PR-11a contained Cys-18, PR-11b and PR-11c contained Cys-34 as a tetrapeptide and a pentapeptide (Table II), respectively.

Fraction PR-18 (500 nmol) was further digested with 1.2 mg of thermolysin in 0.1 M pyridine–acetate, pH 7 at 37 °C for 48 h. The reaction mixture was lyophilized and subjected to diagonal electrophoresis. A pair of peptides (PRT-18a and PRT-18b) moved off the diagonal and contained Cys-27 and Cys-12 (Table II), respectively.

Further indirect evidence for pairing of Cys-18 and Cys-34 came from the application of the Brown & Hartley (1966) diagonal electrophoresis technique to an unfractionated Pronase digest of acid-cleaved inhibitor. PRA-1 (see Table II) appeared to be Cys-Asn-Lys-Pro-Cys-Lys (residues 8–13), PRA-2 corresponded to Cys-Gln-Ala-Cys (residues 24–27), and PRA-3 contained a minor amount of cysteic acid. By difference we conclude that the remaining disulfide bridge is Cys-18-Cys-34.

N-Methylnicotinamide Titrations. *N*-Methylnicotinamide has been proposed as a probe of the environment of tryptophan and tyrosine residues since it binds to these residues only if they are fully exposed to the solvent (Hinman et al., 1976; Deranleau et al., 1975). This reagent appears to form a one-to-one charge-transfer complex with tryptophan and tyrosine residues (Teller & Deranleau, 1976) which can be monitored by an increased absorption in the near-ultraviolet region. Thus, the titration curves of a protein with the reagent can yield the number of tryptophan and tyrosine residues exposed to the solvent.

Figure 4 presents the experimental data obtained for the titration of potato carboxypeptidase inhibitor with *N*-methylnicotinamide chloride (filled circles). The titration was monitored at 350 nm since the extinction coefficients of the charge transfer of the reagent with tryptophan and tyrosine complexes are known at this wavelength (Hinman et al., 1974). There is a distinct curvature to the plot, a conclusion borne out by nonlinear regression analysis. Thus, there are at least two types of sites being titrated, one of which must be a tryptophan, judging from the magnitude of the extinction coefficient (1080 M^{-1} cm^{-1}). This value agrees well with a predicted extinction coefficient of 1010 M^{-1} cm^{-1} (Hinman et al., 1974) for a charge-transfer complex containing one tryptophan and one tyrosine. If the other site were also a tryptophan residue, a much larger extinction coefficient and a linear relationship would have been observed. Theoretical points for titration of one tryptophan and one tyrosine residue, based on reported extinction coefficients, association constants, and the determined protein concentration, are shown as a solid line in Figure 4. The reasonable agreement between experimental and theoretical data points supports the conclusion that the tyrosine and one of the two tryptophan residues are fully exposed to the aqueous environment.

Discussion

Cystine peptides are typically isolated from protein digests obtained by cleavage with strong acids or with pepsin. The carboxypeptidase inhibitor from potatoes proved to be refractory to digestion with pepsin even after partial cleavage with weak acids, whereas partial digestion with strong acids (12 N HCl, 37 °C, 2 days) failed to yield any useful cystine peptides. Cleavage of aspartyl bonds with 0.03 N HCl produced fragments susceptible to digestion with subtilisin or Pronase. Sequenator analysis of the acid-cleaved derivative and amino acid composition indicated that the amino-terminal pentapeptide (residues 1–5) and the aspartyl dipeptide (residues 16–17) had been removed by this treatment. The acid-cleaved derivative was inactive as an inhibitor of carboxypeptidase. The pairing of the disulfide bonds was deduced from the results obtained by digestion of the acid-cleaved inhibitor with subtilisin and confirmed by analysis of the fragment obtained by digestion with Pronase. As indicated in Figure 1, the pairing corresponds to half-cystines 8 to 24, 12 to 27, and 18 to 34. The proof of structure given in the Results section appears to be entirely self-consistent and will not be restated herein.

The carboxypeptidase inhibitor contains 39 amino acid residues (Hass et al., 1975) and three intramolecular disulfide bonds. Such a relatively small molecule must have a rather compact three-dimensional structure with relatively little conformational freedom. If the tendency of hydrophobic residues to occupy internal positions is to be satisfied, the number of allowable configurations would be restricted even further. Visual inspection of the covalent structure shown in Figure 1 would suggest that the cross-linked "core" between half-cystines 8 and 34 would have greater structural constraints than the amino- and carboxyl-terminal regions beyond these residues. In fact, Hass et al. (1976) have presented evidence that the carboxyl-terminal tripeptide segment, -Try-Val-Gly, is in contact with the active sites of carboxypeptidases A and B and that the amino-terminal pentapeptide, Glu-Gln-Ala-His-Asp-, can be removed with little effect on the inhibitory activity of the protein. Thus, the general folding of the polypeptide chain appears to direct the reactive region of the inhibitor toward the solvent where it can interact with carboxypeptidase.

Hass et al. (1976) also found that both tryptophan residues of the inhibitor are unreactive toward the reagents α-hydroxy-5-nitrobenzyl bromide and *o*-nitrophenylsulfenyl chloride, suggesting that both tryptophan residues are buried within the protein molecule. The single tyrosine residue

(residue 37) appears to be exposed. The present charge-transfer data indicate that one tryptophan residue and one tyrosine residue are available to interact with *N*-methylnicotinamide chloride, suggesting that these residues are exposed to the solvent. X-ray crystallographic analysis may be needed to resolve the differences between chemical and charge-transfer evidence.

Acknowledgments

The authors are grateful to Drs. Kenneth A. Walsh and K. Titani for helpful advice throughout this work, to Richard R. Granberg for performing the amino acid analyses, and to Lowell H. Ericsson for sequence analyses.

References

Bennett, J. C. (1967) *Methods Enzymol. 11*, 330.
Brown, J. R., & Hartley, B. S. (1966) *Biochem. J. 101*, 214.
Deranleau, D. A., Hinman, L. M., & Coan, C. R. (1975) *J. Mol. Biol. 94*, 567.
Edman, P., & Begg, G. (1967) *Eur. J. Biochem. 1*, 80.
Fritz, H., Tschesche, H., Greene, L. J., & Truscheit, E., Ed. (1974) *Proteinase Inhibitors*, Springer-Verlag, Berlin.
Hass, G. M., Nau, H., Biemann, K., Grahn, D. T., Ericsson, L. H., & Neurath, H. (1975) *Biochemistry 14*, 1334.
Hass, G. M., Ako, H., Grahn, D. T., & Neurath, H. (1976) *Biochemistry 15*, 93.
Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H., & Walsh, K. A. (1972) *Biochemistry 11*, 4493.
Hill, R. L., & Delaney, R. (1967) *Methods Enzymol. 11*, 4493.
Hinman, L. M., Coan, C. R., & Deranleau, D. A. (1974) *J. Am. Chem. Soc. 96*, 7067.
Hinman, L. M., Coan, C. R., & Deranleau, D. A. (1976) *Biochemistry 15*, 2213.
Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 197.
Laskowski, M., Jr., & Sealock, R. (1971) *Enzymes, 3rd Ed. 3*, 376.
Ryan, C. A. (1971) *Biochem. Biophys. Res. Commun. 44*, 1265.
Ryan, C. A., Hass, G. M., & Kuhn, R. W. (1974) *J. Biol. Chem. 249*, 5495.
Schultz, J. (1967) *Methods Enzymol. 11*, 255.
Smith, I., & Birdenough, M. (1960) in *Chromatographic and Electrophoretic Techniques* (Smith, I., Ed.) Vol. 1, Interscience, New York.
Teller, D. C., & Deranleau, D. A. (1976) *Biochim. Biophys. Acta 421*, 416.
Toennies, G., & Kolb, J. J. (1951) *Anal. Chem. 23*, 823.
Walsh, K. A., Ericsson, L. H., Bradshaw, R. A., & Neurath, H. (1970) *Biochemistry 9*, 219.
Wingard, M., Matsueda, G., & Wolfe, R. S. (1972) *J. Bacteriol. 112*, 940.

Lateral Diffusion of M-13 Coat Protein in Model Membranes†

Lloyd M. Smith, Barton A. Smith, and Harden M. McConnell*

ABSTRACT: A fluorescent derivative of the M-13 phage coat protein (molecular weight 5260) was reconstituted into oriented multilayers and giant liposomes of dimyristoylphosphatidylcholine. The rate of lateral diffusion of the labeled protein in the fluid phase was measured as a function of temperature and found to be comparable to that of lipid probes. The protein was found to have a nonuniform lateral distribution in the solid phase of both types of model membranes. Cardiolipin (0.5 mol %) included in the multibilayers did not have any substantial effect upon the rate of diffusion.

The lateral motion of membrane components is an important factor in cellular immune response (Schlessinger et al., 1976a; Edelman, 1976). In recent years, several techniques have been developed for the measurement of rates of lateral motion of cell surface components (Devaux & McConnell, 1973; Scandella et al., 1972; Sheats & McConnell, 1978; Smith & McConnell, 1978; Kuo & Wade, 1979; Axelrod et al., 1976). Lateral diffusion coefficients have been reported ranging from 10^{-7} cm^2/s to less than 10^{-11} cm^2/s (Devaux & McConnell, 1973; Edidin & Fambrough, 1973; Frye & Edidin, 1970; Peters et al., 1974; Poo & Cone, 1974; Scandella et al., 1972; Schlessinger et al., 1976b; Sheats & McConnell, 1978; Smith & McConnell, 1978; Wu et al., 1977), with cell surface components sometimes appearing immobile on the time scale of the measurements (Peters et al., 1974; Schlessinger et al., 1976b). The interpretation of some of these results is difficult due to the paucity of knowledge about the physical and dynamical properties of membrane proteins. For such interpretations it is crucial to determine the properties of membrane proteins in simple, well-defined model membranes.

We have used fluorescence redistribution after pattern photobleaching (Smith & McConnell, 1978) to measure the rate of lateral diffusion of a fluorescent derivative of the M-13 phage coat protein, reconstituted into both oriented phospholipid multibilayers and giant multilamellar liposomes. The M-13 phage coat protein is a small hydrophobic protein of molecular weight 5260 (Wickner, 1975). It is easily isolable (Makino et al., 1975; Wickner, 1975), its amino acid sequence is known (Asbeck et al., 1969; Nakashima & Konigsberg, 1974), and its properties have been well characterized by a number of research groups (Chamberlain et al., 1978; Hagen et al., 1978; Knippers & Hoffman-Berling, 1966; Makino et al., 1975; Nozaki et al., 1978; Wickner, 1975; Zwizinski & Wickner, 1977). We have measured the temperature dependence of lateral diffusion of the coat protein in the fluid phase of oriented multibilayers and have studied the effect of the phospholipid phase transition. We have also examined the effect of including cardiolipin in the bilayers since evidence

† From the Stauffer Laboratory for Physical Chemistry, Stanford University, Stanford, California 94305. *Received January 29, 1979.* This work has been supported by National Science Foundation Grant PCM 77-23586. L.M.S. is a predoctoral trainee under National Institutes of Health Cellular and Molecular Training Grant GM-7276, and B.A.S. is a National Science Foundation Postdoctoral Fellow.

0006-2960/79/0418-2256$01.00/0