Immunological Studies on the Major Haptenic Peptides from Performic Acid Oxidized Ferredoxin from *Clostridium pasteurianum**

Barbara Kelly and Julia Gerwing Levy†

ABSTRACT: The NH_2-terminal heptapeptide of performic acid oxidized ferredoxin (O-Fd) from *Clostridium pasteurianum* having the amino acid sequence NH_2-Ala-Tyr-Lys-Ile-Ala-Asp-Ser-COOH was shown to have haptenic activity when tested for its ability to bind directly to antiserum prepared against O-Fd and to inhibit complement fixation between O-Fd and its homologous antiserum. Combined studies with this peptide and the COOH-terminal pentapeptide (NH_2-Ala-Pro-Val-Gln-Glu-COOH), previously found to be haptenic, showed that different populations of antibodies were active against each peptide. Equilibrium dialysis experiments using the individual peptides as well as a combination of both indicated that these two peptides account for most of the antibody synthesized in rabbits against O-Fd.

Ferredoxin from *Clostridium pasteurianum* is a single polypeptide of 55 amino acid residues. Previous work in this laboratory indicated that the regions of the ferredoxin molecule containing the 8 cysteine residues (Cys^8 to Cys^{18} and Cys^{37} to Cys^{47}) were probably not critically involved as antigenic determinants, since modification of these residues did not alter critically the reaction of the ferredoxin molecule with its specific antiserum, and that the COOH-terminal chymotryptic peptide (Val^{31} to Glu^{55}) possessed haptenic activity (Nitz *et al.*, 1969). Subsequent solid-phase synthesis of the peptides Val^{31} to Thr^{36} and Pro^{48} to Glu^{55} was carried out and immunological testing showed that the haptenic activity of the COOH-terminal chymotryptic peptide resided in its COOH-terminal octapeptide (Mitchell *et al.*, 1970). Further analysis of the COOH-terminal haptenic group showed that the COOH-terminal pentapeptide was as efficient as the octapeptide in reacting with antiserum to O-Fd[1] (Mitchell and Levy, 1970).

The work reported here entails further studies on the immunological properties of ferredoxin and the identification and testing of a second antigenic determinant. The NH_2-terminal-half of the ferredoxin molecule (extending to Phe^{30}) contains two areas which could be antigenic and cover the regions Ala^1 to Ser^7 and Pro^{18} to Phe^{30}. These two peptides were synthesized and tested for their ability to react with antiserum to O-Fd.

Materials and Methods

The ferredoxin was prepared and purified according to the methods of Tanaka *et al.* (1964) and Mortensen (1964). Performic acid oxidation of the antigen was carried out as previously described (Mitchell *et al.*, 1970). Antiserum to O-Fd was raised in randomly bred albino rabbits by methods described previously (Nitz *et al.*, 1969). Immunized animals received booster immunizations at 6-month intervals with 1.0 mg of O-Fd in saline and 50% complete Freund's adjuvant (Difco). The total volume, which was injected intramuscularly, was in 0.2 ml. Antiserum to O-Fd was purified on a specific immunoadsorbent of bromoacetylcellulose conjugated to O-Fd and prepared according to the methods of Robbins *et al.* (1967).

Solid-phase peptide synthesis was carried out basically according to the method developed by Merrifield (1964) with the modifications suggested by Stewart and Young (1969). The *t*-Boc-amino acids were purchased from Sigma Chemical Co. Two peptides were synthesized; the NH_2-terminal heptapeptide (sequence NH_2-Ala-Tyr-Lys-Ile-Ala-Asp-Ser-COOH) and the 13 amino acid peptide covering the region Pro^{19} to Val^{31} (sequence NH_2-Pro-Val-Asn-Ala-Ile-Ser-Gln-Glu-Asp-Ser-Ile-Phe-Val-COOH). Some modifications in the standard procedure for solid-phase peptide synthesis were essential for the heptapeptide since it contained a tyrosyl residue. The cleavage of the peptide from the resin was effected by passing gaseous HBr (which was scrubbed with trifluoroacetic acid and anisole) through the resin which was suspended in anhydrous trifluoroacetic acid containing a 50-fold molar excess of anisole per estimated mole of tyrosine. The peptide was washed three times with trifluoroacetic acid, dried under reduced pressure, and subsequently washed three times with acetic acid–water (3:1, v/v). The dried peptide was taken up in 10.0 ml of water and extracted three times with ether (to remove the anisole) and the aqueous peptide solution was freeze-dried. This procedure is recommended by Stewart and Young (1969).

Both peptides were subsequently purified chromatographically by the method described by Konigsberg and Hill (1962) using modifications previously described (Mitchell *et al.*, 1970). Following this purification step, the NH_2-terminal heptapeptide was washed with water, dissolved in 2.0 ml of 0.1 N acetic acid, and passed through a 2.0 × 20.0 cm Sephadex G-15 (Pharmacia) column which had previously been equilibrated with 0.1 N acetic acid. This procedure was carried out to free the peptide of any residual traces of anisole. The peptide was then washed three times with water and lyophilized.

Quantitative and Qualitative Analyses. Peptides were checked for purity by testing samples on a Gilson high-voltage electrophoretor at 3000 V for 45 min. Runs were usually made

* From the Department of Microbiology, The University of British Columbia, Vancouver 8, B.C., Canada. *Received January 4, 1971.* This work was supported by a grant from the Medical Research Council of Canada.

[1] Abbreviations used are: O-Fd, performic acid oxidized ferredoxin; Boc, *tert*-butyloxycarbonyl.

TABLE I: Amino Acid Compositions and Molar Ratios of the Synthesized Peptides Covering the Regions Ala[1] to Ser[7] and Pro[19] to Val[31].[a]

	Peptide Ala[1] to Ser[7]			Peptide Pro[19] to Val[31]		
Amino Acid	μmole	Molar Ratio	Residues	μmole	Molar Ratio	Residues
Lysine	0.111	1.34	1			
Aspartic	0.081	0.98	1	0.019	2.11	2
Serine	0.080	0.96	1	0.017	1.89	2
Glutamic				0.009	1.00	1
Proline				0.012	1.33	1
Glycine				0.010	1.11	1
Alanine	0.161	1.94	2	0.011	1.22	1
Valine				0.020	2.22	2
Isoleucine	0.083	1.00	1	0.016	1.78	2
Tyrosine	0.069	0.83	1			
Phenylalanine				0.006	0.67	1

[a] The sequences of the two peptides, respectively, are as follows: NH_2-Ala-Tyr-Lys-Ile-Ala-Asp-Ser-COOH and NH_2-Pro-Val-Asn-Ala-Ile·Ser-Gln-Glu-Asp-Ser-Ile-Phe-Val.

TABLE II: Specific Activity of the N-Acetylated Peptides and Their Relative Binding Properties with Purified Antiserum to O-Fd.[a]

	cpm in Averaged Samples		
Peptide	Sp Act. (cpm/μmole of Peptide)	Sp Antiserum (1.01 mg/ml)	Nonspecific IgG (1.2 mg/ml)
Ala[1] to Ser[7]	7.8×10^6	390 ± 6.5	102 ± 13.3
Pro[19] to Val[31]	5.1×10^6	45 ± 6.3	32 ± 6.0

[a] Counts shown are those obtained from precipitated immunoglobulins (both specific and nonspecific) after incubation with 0.004 μmole of each peptide.

at pH 6.5 in a pyridine–acetate buffer (100 ml of pyridine and 4.0 ml of glacial acetic acid per l.). The paper strips were developed with ninhydrin (5% in acetone) at room temperature. Quantitation of both antigen and peptides was carried out by amino acid analysis on a Spinco Model 120 amino acid analyzer according to the method of Spackman *et al.* (1958).

Acetylation of Peptides. Approximately 4.0 μmoles of each peptide was N acetylated with [^{14}C]acetic anhydride and purified according to the method of Benjamini *et al.* (1965), with the modifications described previously (Mitchell *et al.*, 1970).

Radioactive Measurements. All measurements of ^{14}C-labeled materials were made on a Nuclear-Chicago scintillation counter (Model 725). Samples for counting (these were usually 0.1 ml) were mixed with 8.0 ml of scintillation fluid and were cooled to 4° before counts were made. The scintillation fluid contained 60% toluene, 40% methanol, and 42.0 ml of Liquifluor (New England Nuclear) in a final volume of 1.0 l. Counts were made for 10 min on each of duplicate or triplicate samples.

Hapten Studies. COMPLEMENT FIXATION INHIBITION. The ability of the synthesized peptides to act as haptens was measured by testing their capacity to inhibit complement fixation between O-Fd and its homologous antiserum, as described previously (Gerwing and Thompson, 1968). Negative controls using lysozyme and its homologous antiserum were done simultaneously.

DIRECT BINDING STUDIES. The ability of the peptides to bind directly to purified specific antiserum was tested by a modification of a technique described by Farr (1958). The precise method used here has been described previously (Mitchell and Levy, 1970).

EQUILIBRIUM DIALYSIS experiments were set up using pencil dialysis tubing (Visking, Union Carbide, 1.0 cm) and small screw-cap vials. Purified antiserum to O-Fd, containing 700 μg/ml, was dispensed in 0.5-ml volumes into individual dialysis sacs, which were subsequently tied off. Control sacs containing either phosphate-buffered saline (0.15% NaCl and 0.02% sodium azide in 0.02 M phosphate at pH 7.0) or nonspecific rabbit IgG (at 700 μg/ml) were prepared at the same time. One sac of each type was then suspended in various solutions of the peptides being tested. The tests were run on peptide concentrations between 0.003 and 0.040 μmole per ml. When a combination of two peptides was used, equal concentrations of each peptide were mixed so that the final concentrations did not exceed 0.040 μmole/ml. Dialysis was continued for 5 days at 4° with constant shaking on an Eberbach shaker, at which time equilibrium was reached. Duplicate samples of 0.1 ml were taken from inside each sac and from the outside chamber. The counts per minute for each sample were estimated from 10-min counts, and the average of the duplicate samples was then taken. The results were calculated as r (moles of peptide bound per mole of antibody) and c (concentration of free hapten) and plotted as r/c *vs.* r according to the procedure described by Scatchard (1949).

Results

The two peptides (Ala[1] to Ser[7] and Pro[19] to Val[31]) were synthesized and purified as described. The relevant data regarding sequence and amino acid molar ratios for the purified products are presented in Table I. Both peptides were subsequently tested for their haptenic properties. Initially both peptides were N acetylated and tested for their ability to bind directly to purified antiserum to O-Fd. The data, showing the specific activity of each peptide and their reaction with purified antiserum and nonspecific rabbit IgG, are presented in Table II. The data shown here represent the averaged counts from triplicate tests. While the NH_2-terminal heptapeptide showed significant binding with specific antiserum to O-Fd, the 13 amino acid peptide (Pro[19] to Val[31]) showed no appreciable binding. This latter peptide also lacked the ability to inhibit complement fixation between O-Fd and its homologous antiserum so it was concluded that this region did not constitute a major antigenic determinant, and further work was confined to the NH_2-terminal heptapeptide.

The NH_2-terminal peptide was tested quantitatively for its ability to inhibit complement fixation between O-Fd and its homologous antiserum. Complement fixation inhibition studies were carried out on both the NH_2-terminal heptapeptide and its N-acetylated derivative. No significant difference was observed in their abilities to inhibit the reaction. Since appreciable inhibition was noted in both cases, further studies were carried out to compare the haptenic activity of this pep-

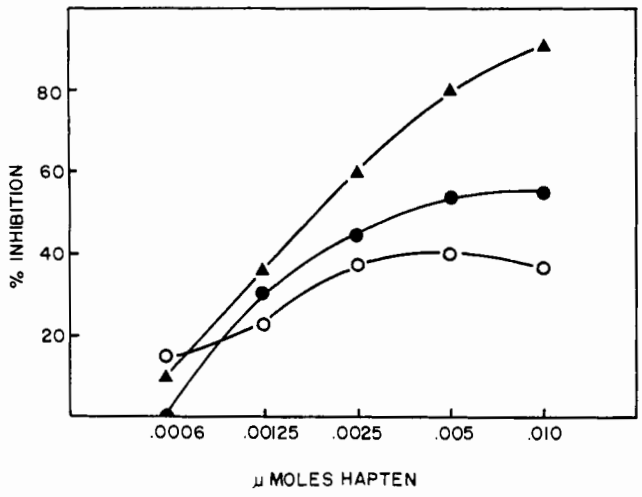

FIGURE 1: Inhibition of complement fixation by the NH_2-terminal heptapeptide (Ala^1 to Ser^7), the COOH-terminal pentapeptide (Ala^{51} to Glu^{55}), and a combination of both peptides when tested against O-Fd and its homologous antiserum. (○) NH_2-terminal heptapeptide, (●) COOH-terminal pentapeptide, and (▲) combined peptides. Complement dilution 1:125, antiserum dilution 1:1200.

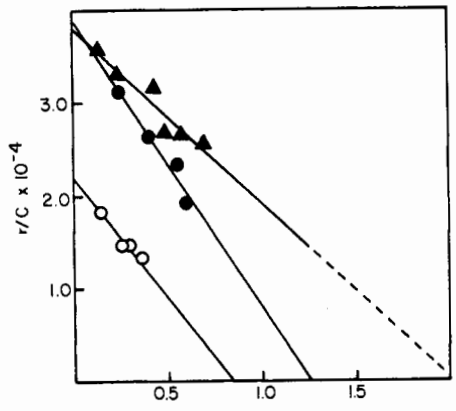

FIGURE 2: The binding of the NH_2-terminal heptapeptide (Ala^1 to Ser^7), the COOH-terminal pentapeptide (Ala^{51} to Glu^{55}), and a combination of both peptides by purified antiserum to O-Fd. (○) NH_2-terminal heptapeptide, (●) COOH-terminal pentapeptide, and (▲) combined peptides. r which is the moles of hapten bound per mole of antibody protein, is plotted *vs.* r/c ($M^{-1} \times 10^{-4}$) in which c is the amount of free hapten present.

tide with that of the COOH-terminal pentapeptide (NH_2-Ala-Pro-Val-Gln-Glu) which had previously been shown to have strong haptenic activity (Mitchell and Levy, 1970). While being slightly less effective in inhibiting complement fixation, the NH_2-terminal peptide did not compete with the COOH-terminal peptide for the same antibody population, since combination of the two peptides in inhibition studies had an enhancing inhibitory effect. The results of these experiments are shown in Figure 1. Nearly total inhibition of complement fixation was noted when 0.01 μmole of each peptide was mixed and used in the test. Control tests using lysozyme and its homologous antiserum were not affected by the presence of comparable concentrations of these peptides.

Equilibrium dialysis studies were carried out on the NH_2-terminal heptapeptide and the COOH-terminal pentapeptide separately and on a mixture of both peptides with antiserum to O-Fd which had been purified on a specific immunoadsorbent. Concentrations of the peptides (which had been N acetylated with [^{14}C]acetic anhydride) ranged between 0.003 and 0.040 μmole per ml. When a mixture of the two peptides was tested, equal concentrations of each peptide were used. The data are shown in Figure 2 as a Scatchard plot and the K^0 values for each test are shown in Table III (these were calculated according to the methods described by Karush, 1962). The data indicate that these two haptenic peptides account for most, if not all, of the antigenic activity of the O-Fd molecule, since a combination of the two peptides appears to bind with specific antibody at 2 moles/mole of antibody (as indicated by extrapolation of the Scatchard plot). Whether or not this observation is valid for more than the two animals' antiserum used here or for other animal species and different immunization procedures remains to be clarified.

Discussion

The work reported here has shown that an antigenic determinant on O-Fd is located in the NH_2-terminal heptapeptide of the molecule since this peptide has the ability to inhibit complement fixation between O-Fd and its homologous antiserum, and to bind directly to purified O-Fd antiserum. The other peptide which was tested (Pro^{19} to Val^{31}) exhibited no immunologic activity and it was concluded that this region did not constitute an antigenic determinant.

Tests involving a combination of the NH_2-terminal heptapeptide and the COOH-terminal pentapeptide (which had previously been shown to constitute an antigenic determinant (Mitchell *et al.*, 1970)) showed that both peptides acted as independent haptens. When complement fixation inhibition tests were run, the NH_2-terminal heptapeptide caused about 40% inhibition, the COOH-terminal pentapeptide about 55% inhibition, and the combination of the two peptides inhibited the complement fixation reaction about 90% (Figure 1). These levels of inhibition were calculated at the titrated optimal level for each system.

The equilibrium dialysis studies reported here indicate that the antibody to O-Fd, raised in rabbits, and purified on a specific O-Fd immunoadsorbent is directed to either one or other of the peptides, since extrapolation of the line obtained on the Scatchard plot (Figure 2) with a combination of the two peptides indicate that 2 moles of hapten is bound per mole of antibody present. These data also indicate that about 60% of the antibody present is directed to the COOH-terminal pentapeptide and about 40% to the NH_2-terminal heptapeptide. The K^0 values obtained in these studies imply that the complex formed between hapten and antibody is not an avid one since the binding constants are low when they are compared to

TABLE III: Association Constant Values from Equilibrium Dialysis Studies Using the NH_2-Terminal Heptapeptide (Ala^1 to Ser^7) and the COOH-Terminal Pentapeptide (Ala^{51} to Glu^{55}) and a Combination of Both with Purified Antiserum to O-Fd.

Peptide Preparation	K^0 ($M^{-1} \times 10^{-4}$)
Ala^1 to Ser^7	1.1
Ala^{51} to Glu^{55}	2.0
Ala^1 to Ser^7 and Ala^{51} to Glu^{55}	1.9

similar measurements on synthetic haptenic groups or to larger haptenic polypeptides which still retain some of their tertiary structure (Fujio *et al.*, 1968). These results are not surprising if one assumes that the molecular conformation of the hapten plays a role in its ability to combine specifically with the antibody. It has been suggested (Crumpton and Small, 1967) that in aqueous solution of a haptenic peptide, at any one time, only a small fraction of the molecules may possess the configuration they did in the whole antigen molecule. Thus, a large percentage of the hapten molecules (those not possessing the native configuration) are precluded from interacting with the antibody molecules.

The data from the equilibrium dialysis studies imply that antibody produced in rabbits to O-Fd is directed either to the COOH-terminal pentapeptide or the NH_2-terminal heptapeptide of the molecule, and that these two regions constitute the main antigenic determinants of O-Fd. It should be emphasized that these results apply only to the experimental conditions reported here, and it is probable that other antigenic determinants are present on the ferredoxin molecule, although under our laboratory conditions we have been unable to detect them.

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Exposed Protein on the Intact Human Erythrocyte*

David R. Phillips† and Martin Morrison‡

ABSTRACT: A method is described which shows that only one molecular weight class of proteins on the human erythrocyte membrane is in an exposed position on the outside of the cell. The method employs lactoperoxidase, a high molecular weight enzyme, to catalyze the iodination of membrane proteins. Since the enzyme iodinates by means of the usual enzyme–substrate complex and cannot diffuse through the membrane because of its size, only those proteins on the surface of the erythrocyte membrane are labeled. In these studies, three states of organization of the erythrocyte membrane have been employed: (1) the intact erythrocyte where only proteins on the surface of the cell were available to lactoperoxidase, (2) the isolated stroma, and (3) the solubilized membrane where all proteins were accessible. Membrane fractions, once iodinated, were solubilized in sodium dodecyl sulfate and fractionated by disc gel electrophoresis. Results showed that only one molecular weight class of proteins with a molecular size of 90,000 is exposed to the outside of the intact erythrocyte while in the conversion of erythrocytes to stroma most membrane proteins became exposed.

Proteins not labeled in these two membrane states could be labeled when free from the membrane indicating that some proteins are not exposed at either membrane surface.

One of the most widely studied membranes from mammalian sources is that of the human erythrocyte. Both qualitatively and quantitatively the lipids, carbohydrates, and proteins have been analyzed in this membrane (Bakermann and Wasemuller, 1967; Rosenberg and Guidotti, 1969; Maddy, 1966; Rouser *et al.*, 1968; Dodge *et al.*, 1963). Other work has evaluated the topography of the membrane with respect to the carbohydrate and lipid moieties (Murphy, 1965; Lenard and Singer, 1968; Eylar *et al.*, 1962; Winzler, 1969).

Although the number and type of peptide units present in the erythrocyte membrane have been defined, determinations of the protein distribution in the membrane has evolved very slowly. The primary reason for the limited amount of knowledge on the spatial arrangement of proteins in this membrane and all membranes is that no technique unequivocally establishes protein position in the membrane. Efforts have been made to determine the arrangement of proteins employing protein-labeling reagents; however, these reagents yield equiv-

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‡ To whom to address correspondence.