Synthesis of Haptenic C-Terminal Octapeptides of Two Cross-Reacting Bacterial Ferredoxin Molecules*

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ABSTRACT: The haptenic properties of two peptides of *Clostridium pasteurianum* ferredoxin were investigated by studying their ability to react with antibody directed against native ferredoxin or performic acid oxidized ferredoxin. These peptides constituted the sequences between Val[31] and Thr[36], and Pro[48] and Glu[55], the latter of which is the C-terminal sequence, and were synthesized by the solid-phase method. The C-terminal octapeptide demonstrated partial haptenic activity when tested for its ability to bind directly with both antisera, or to inhibit either complement fixation or precipitation with these sera and their homologous antigens. The hexapeptide did not demonstrate any haptenic activity. The C-terminal octapeptide of *Clostridium butyricum* ferredoxin (which cross-reacts strongly with antisera directed to *C. pasteurianum* ferredoxin) was also synthesized and demonstrated limited ability to exert haptenic activity with the antisera directed to either native or oxidized *C. pasteurianum* ferredoxin. This sequence differs in only one amino acid at position 53 from the equivalent octapeptide in *C. pasteurianum* ferredoxin.

The ferredoxin of *Clostridium pasteurianum* was shown to be antigenic in rabbits in which it evoked a relatively strong immune response, particularly when the performic acid oxidized derivative was used as the antigen (Nitz *et al.*, 1969). It was also shown that modification of the eight cysteinyl residues by either alkylation, or conversion into cysteic acid did not prohibit cross-reactivity of the modified antigens with antibodies directed to native or performic acid oxidized ferredoxin (O-Fd).[1] These data indicated that the regions of the molecule containing the cysteine residues were probably not critically involved in its antigenicity. Since ferredoxin contains 55 amino acid residues, and the regions containing a high proportion of cysteinyl residues cover the sequences from Cys[8] to Cys[18] and from Cys[36] to Cys[47], the areas involved as antigenic determinants may be somewhat narrowed.

Chymotrypsin splits the ferredoxin molecule into three peptides: the N-terminal dipeptide Ala-Tyr, and the sequences Lys[3] to Phe[30], and Val[31] to Glu[55] (Tanaka *et al.*, 1966). The two major peptides obtained in this way from O-Fd were both found to inhibit the complement fixation reaction between O-Fd and its homologous antiserum (unpublished data) showing that each part of the molecule contained at least one antigenic determinant and could act as a hapten. These two sets of data thus indicated that antigenic regions could involve the sequences Lys[3] to Ser[7], Pro[19] to Phe[30], Val[31] to Thr[36], and Pro[48] to Glu[55] (Figure 1).

The work reported here involves a study of the possible antigenic determinants of the C-terminal-half of the ferredoxin molecule (Val[31] to Glu[55]). The C-terminal octapeptides of both *C. pasteurianum* and *Clostridium butyricum* ferredoxins were synthesized by the solid-phase method and tested for their haptenic activity. These sequences (Pro[48] to Glu[55]) differ in one amino acid only at position 53 (Figure 1). The sequence Val[31] to Thr[36], which is common to both types of ferredoxin was also synthesized and tested in the same manner.

Materials and Methods

Preparation of Antigens. C. pasteurianum and C. butyricum were obtained from Dr. Helen Whitely (Department of Microbiology, University of Washington). *C. pasteurianum* was grown under the conditions described by Tanaka *et al.* (1964) and *C. butyricum* according to the method of Wolfe and O'Kane (1953). The ferredoxins from these cultures were purified by the methods of Mortenson (1964).

Performic acid oxidation of *C. pasteurianum* ferredoxin involved a modification of the methods described by Rall *et al.* (1969) and Tanaka *et al.* (1964). Ferredoxin (30.0 mg) was taken up in 2.5 ml of 8.0 M urea and 0.5 ml of 4.0 M Tris at pH 8.6, to which was added 50.0 mg of 1,10-phenanthroline. After purging the reaction vessel with nitrogen, 200 μl of mercaptoethanol was added and the stoppered reaction flask was kept at room temperature for 6 hr. The solution was brought to pH 3.0 with 6 N HCl, dialyzed exhaustively against 0.01 N acetic acid, and lyophilized. The dialysis step involved some loss of ferredoxin, but it was the most suitable way of removing all the reagents. The dried protein was dissolved in 1.66 ml of formic acid to which was added 3.33 ml of performic acid (3.0 ml of formic acid plus 0.33 ml of 30.0% hydrogen peroxide). The reaction was allowed to continue for 4 hr at -10, after which the material was lyophilized and stored as either a dry powder or as an aqueous solution.

Antisera. Rabbits were immunized to either native or oxidized ferredoxins by methods described previously (Nitz *et al.*, 1969). Serum titers were found to be stable over a 6-month period at which time the animals received a booster inoculation of 1.0 mg of antigen in 0.5 ml of saline mixed with 0.5 ml of complete Freund's adjuvant (Difco). The antigen was administered intramuscularly in the hind leg. Weekly ear bleeds were resumed after a 2-week resting period.

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[1] Abbreviation O-Fd, performic acid oxidized ferredoxin.

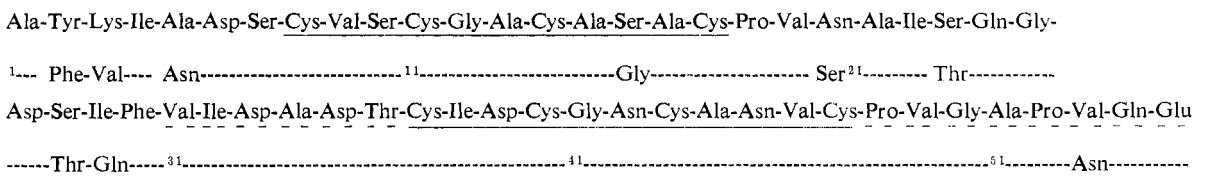

FIGURE 1: The amino acid sequence of *C. pasteurianum* ferredoxin. The second line indicates the sequence of *C. butyricum* ferredoxin and shows only the amino acids which differ from those of *C. pasteurianum* ferredoxin. The unbroken line indicates the cysteine-rich sequences in the molecule. The broken line shows the sequences synthesized in this work.

Purification of Specific Antibody. Specific immunoadsorbents were prepared by complexing bromoacetylcellulose and O-Fd according to the methods of Robbins *et al.* (1967). The adsorbent was prepared in 1.0-g amounts and reacted with total volumes of 75.0 ml of immune serum at a time, so that a considerable molar excess of antigen was always present to ensure maximum binding of specific antibody. Antibodies against both native ferredoxin and O-Fd were purified using this immunoadsorbent, since it was previously shown that the antiserum to native ferredoxin reacted more strongly with O-Fd than it did with the native molecule (Nitz *et al.*, 1969). The adsorbed antibody was eluted with 0.1 N acetic acid and dialyzed exhaustively at 4 against 0.1 M NaCl–0.01 M Tris-HCl at pH 7.0. It was stored in this buffer at −15 in concentrations of 0.5 mg/ml.

Solid-Phase Peptide Synthesis. The following peptides were synthesized by the basic method of Merrifield (1964): Val-Ile-Asp-Ala-Asp-Thr (residues 31–36 in *C. pasteurianum* and *C. butyricum* ferredoxins), Pro-Val-Gly-Ala-Pro-Val-Gln-Glu (residues 48–55 in *C. pasteurianum*, and Pro-Val-Gly-Ala-Pro-Asn-Gln-Glu (residues 48–55 in *C. butyricum*). The modification of the original methods and precise procedures recommended by Stewart and Young (1969) were used throughout. The chloromethylated copolysterene divinylbenzene resin was obtained from Bio-Rad and the *t*-Boc-amino acids were obtained from either Mann Research Laboratories or Sigma Chemical Co.

Syntheses were carried out in each case on 1.0 g of resin, and amino acids were reacted at each step in a 3.5 molar excess, with the exception of the addition of Gln and Asn (in the form of *p*-nitrophenyl esters), in which a fivefold excess was used. Cleavage of the peptides from the resin was performed by passing gaseous HBr through the resin in anhydrous trifluoroacetic acid for 90 min at 25. Subsequent hydrogenation was not carried out since the *O*-benzyl esters on Asp, and Glu, and Thr are removed during the cleavage step.

Peptide Purification. Following their cleavage from the resin, the synthesized peptides were lyophilized and taken up in *N*-methylmorpholine–acetic acid buffer at pH 8.4 (0.2 M in acetic acid), and chromatographed on Dowex 1-X8 (200–400 mesh, Bio-Rad) according to a modification of the methods of Konigsberg and Hill (1962). The resin, in the acetate form, was poured into a 1 × 20 cm column and equilibrated in the *N*-methylmorpholine–acetic acid buffer at pH 8.4. After application of the sample to the column, a gradient was run between 100 ml of this buffer and 100 ml of pyridine–acetate buffer at pH 6.56 (325 ml of pyridine and 5.6 ml of glacial acetic acid per l.). At the completion of the gradient, pyridine–acetate buffer at pH 4.05 (162 ml of pyridine and 560 ml of acetic acid per l.) was applied to the column to complete the elution. Columns were run at room temperature at 60 ml/hr and 5.0-ml fractions were collected on an LKB fraction collector. After alkaline hydrolysis, quantitative ninhydrins were carried out on 0.2-ml aliquots of each fraction, according to the method of Hirs *et al.* (1956).

Protein and Peptide Analysis. Purity of the peptide samples was checked by high-voltage paper electrophoresis in pyridine–acetate buffer at pH 6.5 (100 ml of pyridine and 4.0 ml of acetic acid per l.) on a Gilson high-voltage electrophoretor. Electrophoretograms were developed with ninhydrin (5% in acetone). Quantitation and analysis of both antigens and peptides were performed by amino acid analysis on a Spinco Model 120 amino acid analyzer according to the method of Spackman *et al.* (1958).

Radioactive Measurements. All measurements of ^{14}C-labeled materials were made on a Nuclear-Chicago scintillation counter (Model 725). Aliquots of solutions to be counted were mixed with 8.0 ml of scintillation fluid and cooled at 4° before counts were made. The scintillation fluid contained 60% toluene, 40% methanol, and 42.0 ml of Liquiflour (New England Nuclear Corp.) in a final volume of 1 l.

Acetylation of Peptides. The [1-^{14}C]acetic anhydride (5 mCi/mmole) used in these reactions was obtained from New England Nuclear Corp. in break seal tubes containing 0.1 mCi. The container was frozen in a Dry Ice–acetone bath and 2.0 ml of benzene was placed in the tube over the vacuum seal. The seal was broken and the benzene was automatically drawn into the tube thus trapping the acetic anhydride.

Peptides to be acetylated were dissolved at concentrations of between 5.0 and 10.0 μmoles in 2.0 ml of 1.0 M sodium acetate at pH 8.0 and cooled to 4. As recommended by Fujio *et al.* (1968), 0.5 ml of the benzene solution of labeled acetic anhydride was carefully layered on top and the acetylation reaction was permitted to continue for 12 hr at 4. The aqueous layer was then applied to a 2 × 20 cm Sephadex G-15 column which had previously been equilibrated with 50% acetic acid. Elution in 50% acetic acid was carried out at room temperature. The flow rate was adjusted to 6.0 ml/hr and 2.0-ml fractions were collected from which 0.1-ml samples were taken for counting. The fractions containing peptide were dried, washed with water, and dried again under vacuum. Quantitation of the acetylated peptides was carried out by amino acid analysis.

Immunological Assays. EQUILIBRIUM DIALYSIS. Equilibrium dialysis experiments were set up using pencil dialysis tubing (Visking-Union Carbide, 1 cm) and small screw-cap vials. Purified antisera to both native ferredoxin and O-Fd (pre-

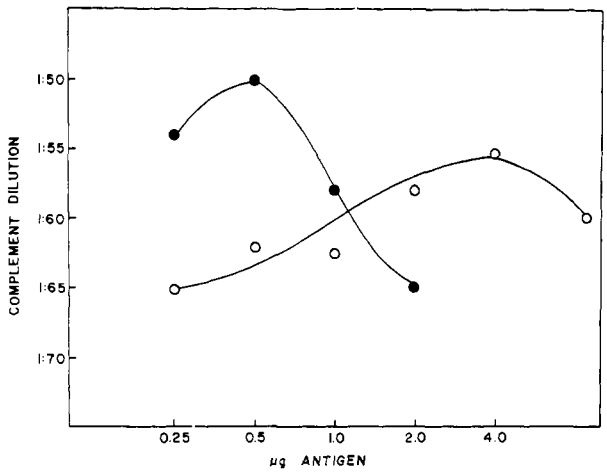

FIGURE 2: The complement fixation reaction between *C. pasteurianum* ferredoxin and its homologous antiserum (●—●) and the cross-reaction of *C. butyricum* ferredoxin with the same serum (○—○). Serum dilution was 1:40.

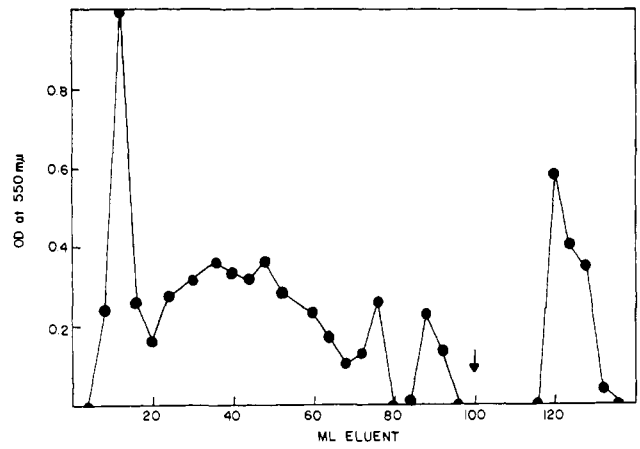

FIGURE 3: The elution profile of the hexapeptide from Dowex 1-X8. The arrow indicates the time of the buffer change after completion of the gradient. The peak eluting at 120 ml contained the hexapeptide.

pared by immunoadsorption with O-Fd), and containing between 300 and 400 μg per ml in 1.0-ml volumes, were placed in individual dialysis sacs, and suspended in solutions of the various acetylated peptides which were at concentrations of 0.001, 0.002, and 0.004 μmole per ml in phosphate-buffered saline (0.15% NaCl and 0.02% sodium azide containing 0.02 M phosphate at pH 7.0). Controls entailed dialysis of nonespecific rabbit IgG at the same concentrations as the specific antisera and of phosphate-buffered saline against the various peptide preparations. The dialysis was continued for 4 days at 4° with constant shaking on an Eberbach shaker, at which time equilibrium had been reached. Samples of 0.1 ml were taken from inside and outside the dialysis sacs for measurement of radioactivity. The counts per minute were estimated from 10.0-min counts which were carried out on each sample.

COMPLEMENT FIXATION. The procedures by which complement fixation was used for either direct titration of immune serum, or as a means of detecting hapten inhibition by the various peptides has been described previously in detail (Gerwing and Thompson, 1968).

QUANTITATIVE PRECIPITIN REACTION. The quantitative precipitin reaction was also carried out as described previously (Gerwing and Thompson, 1968). When this method was used to test hapten inhibition, a modification of the previously described procedure was applied. Varying quantities of the test hapten in 0.1 ml of 0.9% NaCl (between 500.0 and 5.0 μg) were mixed with constant amounts of antiserum (0.5 ml) to O-Fd, and the tubes were incubated for 1 hr at 37. Antigen at a concentration previously estimated to be slightly on the excess side of the optimal proportions range, was then added in a volume of 0.5 ml and incubation at 37 was continued for another hour. The precipitates thus formed were centrifuged, washed in saline, dissolved in 0.1 N NaOH, and read for 280-mμ absorbance on a Beckman DBG spectrophotometer. Each test was done in triplicate and per cent inhibition was calculated on the differences in the averaged readings when compared with values from control tubes containing 0.1 ml of saline instead of peptide.

Results

Antiserum prepared against native ferredoxin from *C. pasteurianum* was titrated by complement fixation with both the homologous antigen and the ferredoxin from *C. butyricum*. The results are shown in Figure 2 and indicate relatively strong cross-reactivity. This observation was verified when the heterologous ferredoxin was tested with antiserum to O-Fd at the precipitin level, indicating that more than one antigenic determinant on the molecule bore a close resemblance to those on the *C. pasteurianum* ferrodoxin. This is not surprising since many areas of sequence homology exist between the two molecules (Figure 1).

In an attempt to determine the regions responsible for antigenicity and cross-reactivity of the two molecules, three peptides were synthesized by the solid-phase method. These peptides included the sequence Val^{31} to Thr^{36}, which is a sequence identical in the two molecules, and Pro^{48} to Glu^{55} which varies at position 53 in which there occur Val and Asn in the ferredoxins of *C. pasteurianum* and *C. butyricum*, respectively.

The synthesized peptides were cleaved from the solid-phase resin, washed, and applied to Dowex 1-X8 columns for purification. The elution profile for the hexapeptide and the C-terminal octapeptide from *C. pasteurianum* are shown in Figures 3 and 4. The latter is very similar to the elution profile obtained by the chromatography of the octapeptide from *C. butyricum*.

The peak containing the desired peptide was identified by amino acid analysis of aliquots from the various ninhydrin-positive fractions, the best amino acid ratios being obtained in the peak eluting at 120.0 ml for the hexapeptide and in the peaks eluting between 10.0 and 20.0 ml for the two octapeptides (Table I). Since high-voltage electrophoresis of these fractions demonstrated only one major ninhydrin-positive component, it was assumed that these fractions contained the desired peptide in a relatively pure state. A summary of the yields obtained from the various peptides is shown in Table II. In all cases, much less than the theoretical total yield was obtained in terms of attachment of the C-terminal amino

TABLE I: Amino Acid Compositions and Molar Ratios of Three Peptides Prepared by the Solid-Phase Synthetic Method.[a]

Amino Acid	Hexapeptide			Octapeptide *C. pasteurianum*			Octapeptide *C. butyricum*		
	μmole	Molar Ratio	Residues	μmoles	Molar Ratio	Residues	μmole	Molar Ratio	Residues
Aspartic	0.170	1.42	2				0.146	0.91	1
Threonine	0.113	0.94	1						
Glutamic				1.121	2.19	2	0.314	1.95	2
Proline				1.033	2.02	2	0.332	2.06	2
Glycine				0.476	0.93	1	0.171	1.06	1
Alanine	0.140	1.18	1	0.547	1.07	1	0.164	1.02	1
Valine	0.144	1.20	1	1.025	2.00	2	0.161	1.00	1
Isoleucine	0.133	1.11	1						

[a] Sequences of the peptides are as follows: hexapeptide: Val-Ile-Asp-Ala-Asp-Thr, *C. pasteurianum* octapeptide; Pro-Val-Gly-Ala-Pro-Val-Gln-Glu, *C. butyrcium* octapeptide; Pro-Val-Gly-Ala-Pro-Asn-Gln-Glu.

acid to the resin which had a milliequivalence of 0.72, and in terms of the total yield of purified peptide, when compared to the amount of C-terminal amino acid attached.

Samples of each synthesized peptide were acetylated with [1-^{14}C]acetic anhydride and freed from labeled acetic acid by passage of the acetylation mixture through Sephadex G-15. Quantitation of the peptides after elution, washing, drying, and amino acid analysis yielded preparations containing 3.4×10^6, 3.5×10^6, and 1.8×10^6 cpm per μmole for the hexapeptide, the *C. pasteurianum* octapeptide, and the *C. butyricum* octapeptide, respectively.

Pooled rabbit antisera to native ferredoxin and O-Fd were purified by attachment of specific antibody to a bromoacetyl-cellulose immunoadsorbent covalently linked to O-Fd The two purified preparations titered by complement fixation at 1:160 and 1:900 for ferredoxin and O-Fd, respectively, which indicated approximately 90% recovery of specific antibody activity. The concentration in each preparation was 420 μg/ml for the O-Fd antibody and 240 μg/ml for the ferredoxin antibody.

The three acetylated peptides in phosphate-buffered saline were dialyzed against the purified antibody preparations as well as against nonspecific rabbit IgG at 300 μg/ml, and against phosphate-buffered saline. The only peptide showing significant binding with the ferredoxin antisera was the *C*-terminal octapeptide of *C. pasteurianum* ferredoxin which bound significantly with both antibody preparations, although binding with the O-Fd antibody was slightly greater. The K_0 (1/mole) was calculated as 6.1×10^4 for O-Fd antiserum and 4.3×10^4 for ferredoxin antiserum. These values were estimated from calculations made at 0.004, 0.002, and 0.001 μmole per ml of the acetylated octapeptide. The equivalent peptide from *C. butyricum* or the hexapeptide common to both molecular species did not show any binding with either antibody under the test conditions used here.

Further tests were carried out on the synthesized peptides to determine whether or not other types of assay would verify the observations made using equilibrium dialysis. The peptides were tested for their ability to inhibit complement

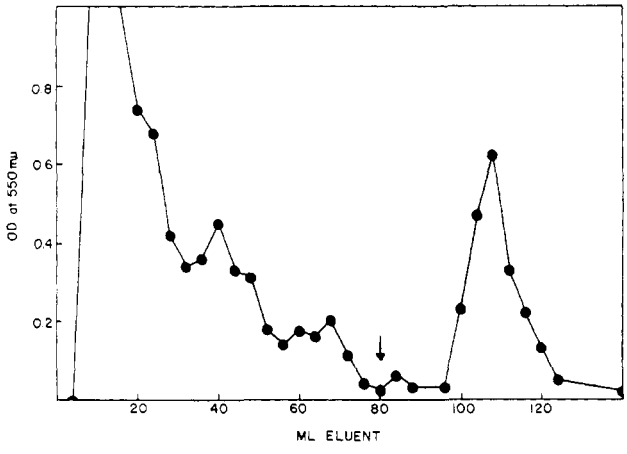

FIGURE 4: The elution profile of peptide Pro^{48} to Glu^{55} of *C. pasteurianum* ferredoxin. The arrow indicates the time of the buffer change after completion of the gradient. The peak eluting between 10 and 20 ml contained the octapeptide.

TABLE II: Calculations of the Yields of Amino Acids and Peptides at Various Stages in the Solid-Phase Synthesis of the Peptides.

	Hexapeptide	Octapeptide *C. pasteurianum*	Octapeptide *C. butyricum*
Attachment (μmoles/mg of Resin)	0.112	0.267	0.287
Per cent of theoretical	15.5	36.8	39.9
Yield of peptide (μmoles)	11.7	100.0	32.3
Per cent of theoretical (calculated from the attachment figure)	10.5	37.5	11.3

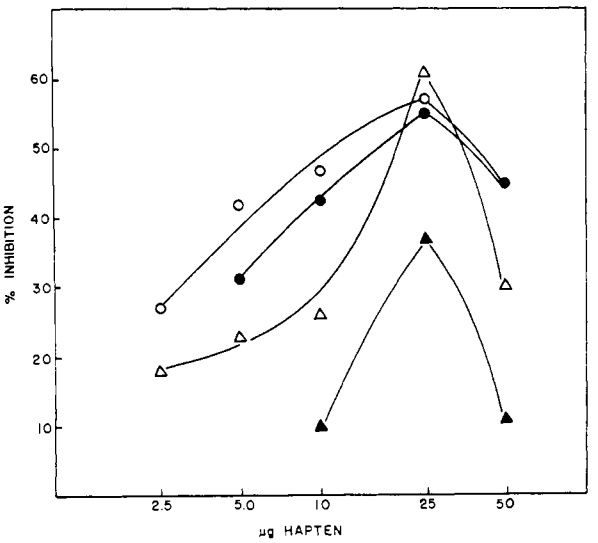

FIGURE 5: Inhibition of complement fixation by the octapeptide of *C. pasteurianum* ferredoxin when tested against native ferredoxin and O-Fd and their homologous antisera. (O—O, ●—●) Dilutions of 1:100 and 1:125 of complement with O-Fd and anti-O-Fd. (Δ—Δ, ▲—▲) Same dilutions of complement with native ferredoxin and its homologous antiserum.

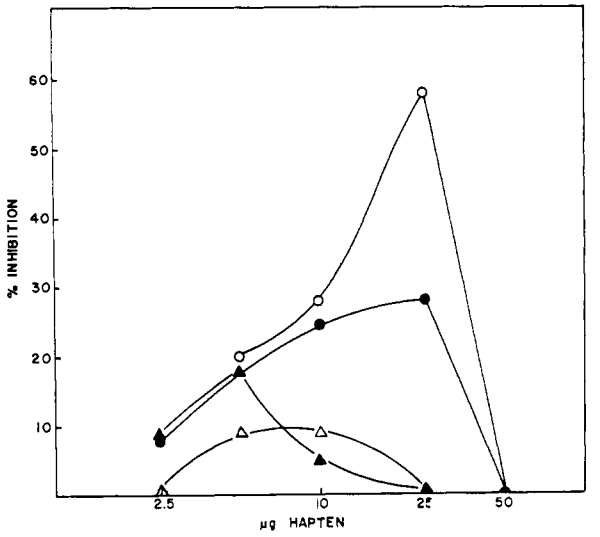

FIGURE 6: Inhibition of complement fixation by the octapeptide of *C. butyricum* ferredoxin when tested against native ferredoxin and O-Fd and their homologous antisera. (O—O, ●—●) Dilutions of 1:100 and 1:125 of complement with O-Fd and anti-O-Fd. (Δ—Δ, ▲—▲) Equivalent dilutions of complement with native ferredoxin and its homologous antiserum.

fixation between the antisera to O-Fd and native ferredoxin and their homologous antigens. The peptides were tested over a range of 50.0–2.5 μg. The results showed that the C-terminal octapeptide of *C. pasteurianum* over this range was a powerful inhibitor of the reactions with both systems. Representative results are shown in Figure 5 and show the ability of the peptide to inhibit the complement fixation reaction at the two dilutions of complement permitting in the range of 50% hemolysis for the control systems not containing the peptide. Per cent inhibition was calculated on the difference in per cent hemolysis between the control tubes and the tubes containing peptide when they were compared with the serum control at these concentrations of complement. The results obtained with the C-terminal octapeptide of *C. butyricum* are shown in Figure 6, and it can be seen that when this method of assay is used, cross-reactivity of this peptide with the *C. pasteurianum* antisera can be demonstrated. Although the degree of inhibition was not high in all instances, these results were reproducible and consistent. A negative control using lysozyme and its homologous antiserum showed that the effect with the octapeptides was specific since no inhibition was observed with this system.

The hexapeptide, when tested for its inhibitory capacity by the complement fixation assay system, showed no ability to inhibit the specific immune reaction.

The ability of the two octapeptides to inhibit the precipitin reaction between O-Fd and its homologous antiserum was also tested. The range of hapten concentration used was between 500 and 2.5 μg. A representative set of results are shown in Figure 7. It can be seen that even at these high concentrations of hapten, the maximum range of inhibition has not been reached and the levels of inhibition are still quite low. However, these results were reproducible within 0.5% when comparisons were made between averaged triplicate samples from separate tests. A negative control using lysozyme and its homologous antiserum showed that this effect was specific.

Discussion

The observation that the ferredoxin of *C. butyricum* showed strong cross-reactivity with the antisera prepared against *C. pasteurianum* ferredoxin and its performic acid oxidized derivative was not surprising since the sequences of these two molecules are very similar and differ at only 9 positions out of the total 55 (Figure 1). The data obtained, showing that

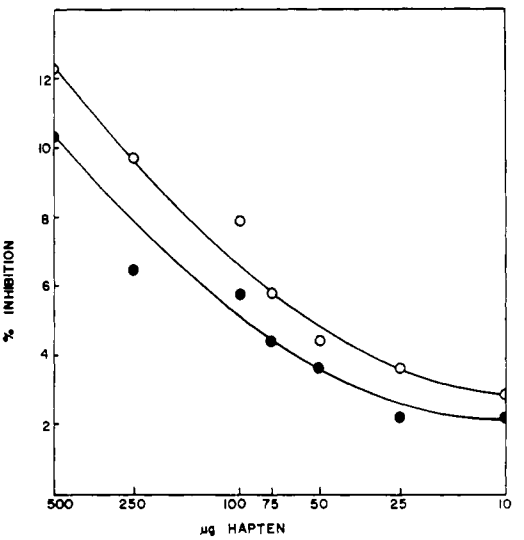

FIGURE 7: Inhibition of precipitation with O-Fd and its homologous antiserum in the presence of varying amounts of the octapeptides from *C. pasteurianum* and *C. butyricum* ferredoxins. (●—●) *C. butyricum* octapeptide; (O—O) *C. pasteurianum* octapeptide.

cross-reactivity as opposed to total reactivity existed between the two molecules indicated that at least some of the regions responsible for antigenicity were those in which amino acid differences existed.

Earlier observations made by us (Nitz *et al.*, 1969) that the regions of the ferredoxin molecule containing cysteine residues (Cys^{8} to Cys^{18}, and Cys^{37} to Cys^{47}) were probably not critically involved as antigenic determinants narrowed the possible antigenic regions considerably. The C-terminal-half of ferredoxin (Val^{31} to Glu^{55}) can be obtained by chymotryptic cleavage of the molecule and subsequent separation by paper electrophoresis. This portion of the molecule acts as an inhibitor of complement fixation between O-Fd and its homologous antiserum (unpublished data). This peptide presents two likely regions of antigenicity, Val^{31} and Thr^{36}, a sequence common to both *C. pasteurianum* and *C. butyricum* ferredoxin, and the C-terminal octapeptide Pro^{48} to Glu^{55} which varies at position 53 with a Val substituted for Asn.

The synthesis of these sequences enabled us to study their activity as haptens. It was concluded that the hexapeptide exerted no haptenic effect since it was ineffective as a hapten when tested for inhibition of complement fixation with the antisera to O-Fd and native ferredoxin and their homologous antigens. It also showed no specific binding with these antisera when it was N acetylated and tested for this by equilibrium dialysis.

The octapeptide for *C. pasteurianum*, on the other hand, showed a marked ability to inhibit complement fixation with both antisera used, and the N-acetylated derivative showed significant and comparable binding with antibodies purified by immunoadsorbance with O-Fd, to both O-Fd and native ferredoxin. It should be pointed out that following the acetylation of the peptides, no attempt was made to separate acetylated from nonacetylated molecules because the amount of labeling obtained was sufficient to study binding at the levels of hapten used in the studies (between 0.004 and 0.001 μmole per ml of hapten). Therefore it is possible that a greater degree of binding occurred than was observed, since equilibrium dialysis measurements were made solely against the acetyl peptide, although acetylation appeared to have been quite efficient (peptides contained between 1.8×10^{6} and 3.5×10^{6} cpm per μmole, and the specific activity of the acetic anhydride was 5 μCi/μmole).

The antibodies to native ferredoxin were purified on a bromoacetylcellulose immunoadsorbent attached to O-Fd. Thus this antibody population, although it contained virtually all the antibodies that react with native ferredoxin (as determined by the complement fixation titer of the purified material when compared with the original titer of the serum with native ferredoxin), also, no doubt, contained antibodies which react preferentially with the O-Fd than it did with the homologous system, and it was suggested at that time that this might be because ferredoxin, after inoculation into rabbits, becomes modified to such an extent that antibodies stimulated by its administration react better with a denatured form of the molecule. Thus, the observation that purified Fd antiserum bound the octapeptide to almost the same extent as the O-Fd antiserum, could reflect the activity of antibodies to denatured antigen. However, the observation that the octapeptide could inhibit the complement fixation reaction between native ferredoxin and its antiserum indicate that indeed this peptide exhibits haptenic activity in the native system.

The octapeptide representing the C terminal of *C. butyricum* ferredoxin exerted haptenic activity when tested by complement fixation and precipitin reactions, although to a considerably lesser extent than the homologous octapeptide in most cases. However, we were unable to demonstrate the direct binding of this peptide with either the native ferredoxin or the O-Fd antibodies. This might be due to the fact that the acetylated derivative was not separated from unlabeled material, and a limited degree of binding (which one would expect) could be masked by the presence of unlabeled material. This possibility is currently under investigation. It is interesting to note that the single amino acid change at position 53 in which Asn is substituted for Val in the *C. butyricum* molecule is sufficient to reduce markedly the immunological activity of this peptide with antisera against the *C. pasteurianum* ferredoxin molecule but does not appear to be so critical in reactions involving O-Fd and its homologous antiserum (Figures 6 and 7).

In conclusion, it would appear that the C-terminal octapeptide of the ferredoxin molecule constitutes a region of antigenicity. The participation of the C-terminal region of this antigen is interesting in view of the observations on some other molecules, namely, sperm whale myoglobin and egg-white lysozyme (Crumpton and Wilkinson, 1965; Fujio *et al.*, 1968; Young and Leung, 1969), in which active participation of the C-terminal regions have also been implicated as antigenic determinants. This, however, is not found universally since there are many proteins in which the C terminal does not appear to be antigenic. Further work on the bacterial ferredoxins will attempt to elucidate more concisely the exact number of amino acids in the octapeptide essential for binding with specific antibody.

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