Localization of a Highly Immunogenic Region of Carboxypeptidase A Recognized by Three Different Monoclonal Antibodies and Their Use in the Detection of Subtle Conformational Alterations in This Enzyme Region

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ABSTRACT: Three murine monoclonal antibodies (mAb 100, 104, and 121) elicited against carboxypeptidase A (CPA) were prepared and characterized. All three mAbs recognize the same or partially overlapping sites of CPA. This is corroborated by the lack of antibody additivity in the ELISA assay carried out in the presence of pairs of mAbs, the similarity in molecular weight of the immunocomplex formed between CPA and one of the mAbs in the presence of another, and also a competition experiment in which one of the mAbs was labeled enzymatically. The three mAbs do not affect the enzymatic activity of CPA. Even at high concentrations, they do not recognize carboxypeptidase B (CPB) in spite of the similar tertiary structure and the 50% homology in amino acid sequence with CPA. This antigenic determinant is located on one of the four cyanogen bromide fragments of CPA. On the basis of the known sequences of the two enzymes, criteria which predict high antigenicity, and experimental data using synthetic peptides, such a determinant was found to be located within the amino acid sequence from residues 209 to 218 of the CPA molecule. The mAbs prepared detect conformational alterations in the above enzyme epitope when the enzyme is exposed to various conditions. The binding of the mAbs to CPA adsorbed onto a polystyrene plate is characterized by apparent binding constants higher by 1 or 2 orders of magnitude than those characterizing the interaction of the mAbs with CPA in solution. The mAbs also readily detect both conformational alterations of CPA on treatment with urea and subtle, reversible conformational alterations on removal of zinc from the active site of the enzyme.

The use of monoclonal antibodies (mAbs)[1] for the detection of conformational alterations occurring under well-defined conditions in the corresponding protein antigens was recently reviewed by Katchalski-Katzir and Kenett (1988). In the present paper, we described the use of mAbs directed toward carboxypeptidase A (CPA) for the detection of subtle conformational changes occurring within the enzyme molecules on adsorption onto polystyrene plates, after removal of zinc from the active site of the enzyme, and in treatment with urea. The three mAbs chosen in this study, mAb 100, mAb 104, and mAb 121, of which preparation and characterization are described, bind to the same or partially overlapping epitope(s) of CPA with relatively high binding constants, without affecting its enzymic activity. In previous studies (Solomon et al., 1984b) using the above characteristics of these mAbs, a novel method for the preparation of the highly active immobilized enzyme via monoclonal antibodies was developed.

The region on CPA recognized by the mAbs was characterized, and alteration in their binding constants with CPA correlated with corresponding conformation changes occurring in the enzyme under well-specified conditions.

In principle, antigenic determinants are divided into two structural categories: sequential and assembled topographical determinants (Benjamin et al., 1984).

A number of approaches have searched for and defined antigenic sites. X-ray crystallography is the most potent method, and recently the first immunocomplex of a protein (Fab D1.3; hen egg white lysozyme) has been analyzed to 2.8-Å resolution (Amit et al., 1985). The determination of the immunoreactivity of antibodies with short peptides corresponding to known regions of the protein molecule is another approach, applicable to continuous antigenic sites. Such peptides may be obtained either by fragmentation (proteolytic or chemical) of the protein or by chemical synthesis. When peptides are to be synthesized, they are often chosen on the basis of hydrophilicity (Hopp & Woods, 1981; Kyte & Doolittle, 1982), segmental flexibility (Westhof et al., 1984), accessibility (Novotny et al., 1986), and other pertinent data characterizing the protein antigen in order to locate structures of high antigenicity.

We have described the identification of the sequential antigenic region of carboxypeptidase A recognized by the three monoclonal antibodies mAb 100, mAb 104, and mAb 121 using the immunochemical approach. On the basis of the experimental data, using synthetic peptides as well as prediction criteria, the antigenic determinant common to the above antibodies was found to be located within amino acid sequence 209–218 of the CPA molecule.

Materials and Methods

Enzymes. CPA was obtained as an aqueous crystal suspension (Sigma Chemical Co., St. Louis, MO). The crystals were washed with double-distilled water, centrifuged, and dissolved in 0.05 M Tris-HCl/0.5 M NaCl buffer, pH 7.5. Insoluble material was removed by centrifugation. The enzyme concentration was derived from the absorbance at 278

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[1] Abbreviations: APase, alkaline phosphatase; BSA, bovine serum albumin; CPA, carboxypeptidase A; CPB, carboxypeptidase B; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; SPA, Sepharose–protein A; PBS, phosphate-buffered saline.

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nm, measured by a Perkin-Elmer Model 550-S spectrophotometer, using a molar absorptivity of 6.42×10^4 M^{-1} cm for native CPA (Johansen & Vallee, 1975). Protein concentration was also determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard. The enzymic activities of CPA and its derivatives were determined spectrophotometrically at 254 nm using either 1 mM hippuryl-L-phenylalanine or hippuryl-DL-β-phenyllactic acid in 0.5 M NaCl/0.05 M Tris-HCl, pH 7.5, according to Whitaker et al. (1966).

Apocarboxypeptidase A and reconstituted enzyme were prepared according to Auld and Vallee (1970).

Preparation of Monoclonal Antibodies to CPA. Mouse monoclonal antibodies to CPA were prepared in collaboration with Bio-Yeda, Israel, following the fusion techniques of Kohler and Milstein (1975). Hybridomas which produced supernatants with relatively high titers were selected, cloned, and used for the preparation of the corresponding ascitic fluids. The monoclonal antibodies present in these ascitic fluids were then isolated by precipitation with 50% ammonium sulfate (Goding, 1980) and their binding constants with CPA determined by means of a modified ELISA technique (Ball et al., 1982). Three monoclonal antibodies, mAb 100, 104, and 121, which interact with CPA with high binding constants (ELISA assay, $K_{app} = 10^9$ M^{-1}), were chosen for further study.

The immunochemical nature of the selected mAbs was established by an immunodiffusion Ouchterlony test (Ouchterlony & Nilsson, 1978) using goat antibodies against the various mouse immunoglobulin isotypes (Meloy, Springfield, VA). mAbs 100 and 121 belong to subclass IgG1, while mAb 104 represents subclass IgG2a.

Purification and Characterization of mAbs. mAbs 100, 104, and 121 were isolated and purified by affinity chromatography on protein A–Sepharose (SPA) from the corresponding ascitic fluids according to Ey et al. (1978). Protein concentrations were determined according to Bradford (1976) using normal murine IgG as a standard; 1.0, 1.0, and 0.8 mg of each were detected in the 1-mL solutions of mAbs 100, 104, and 121, respectively.

The enzyme (2 μg in 2 μL of 0.05 M Tris-HCl/0.5 M NaCl buffer, pH 7.5) was incubated for 1 h at room temperature with increasing amounts of purified mAbs (10–100 μg in 100 μL in the same buffer). The peptidase and esterase activities of the incubation mixtures were assayed as described above.

ELISA Assays. The antigen-coating solutions (100 μL), containing native or denatured CPA (10–25 μg/mL) in PBS, pH 7.4, were incubated overnight at 4 °C in a polystyrene ELISA plate (Costar, Cambridge, MA). Diluted ascitic fluid (0.1 mL) containing the desired mAb (1:2000–1:18000 v/v in PBS) was added and incubated at 37 °C for 1 h. The apparent amount of mAb bound was determined with β-galactosidase-linked $F(ab)_2$ fragments of sheep anti-mouse IgG (Amersham International, U.K.) (Engvall & Perlmann, 1971; Ball et al., 1982).

Prior to coating, CPA denaturation was carried out as follows: the enzyme (1 mg) was incubated in 8 M urea (1 mL, pH 7.0, for 1 h at room temperature). Aliquots of 10–25 μL were withdrawn and diluted in 1 mL of coating buffer (PBS, pH 7.4, containing 10–25 μg/mL BSA).

Determination of Apparent Binding Constants (K_{app}) Characterizing the Interaction of CPA with mAbs. The apparent constants characterizing the binding of mAbs 100, 104, and 121 to CPA were determined (1) in a heterogeneous ELISA system in which CPA is adsorbed onto a polystyrene plate and (2) in a homogeneous system in which both CPA and the corresponding mAbs are free in solution.

(1) In the heterogeneous ELISA system, different dilutions (1:2000–1:18000 in PBS, pH 7.4) of the ascitic fluids or of the final purified mAb solutions were employed. Their apparent binding constants were determined according to the procedure of Pinckard and Weir (1978), i.e., as derived from the reciprocal of the free monoclonal antibody concentration at which 50% of maximal binding to CPA was achieved.

(2) In the homogeneous system, CPA (0.2–2.0 μg) in 0.8 mL of 0.1 M phosphate buffer, pH 8.0, was incubated for 1 h at room temperature with a constant amount of monoclonal antibody (2.0 or 2.5 μg) in 150 μL of PBS, pH 7.4. SPA (5.0 μL) containing 10 μg of protein A was then added to the reaction mixture. The suspension was incubated for 1 h at 37 °C with agitation, while ensuring constant floating of the solid material in solution. The reaction suspension was centrifuged, and residual enzyme activity in the supernatant was measured.

The experimental data were expressed as Scatchard plots (Scatchard, 1948).

Sucrose Gradient Ultracentrifugation Experiments. A linear sucrose density gradient was prepared ranging from 5 to 20% sucrose in PBS, pH 7.4. To 12 mL of the solution prepared was added 0.2 mL of solution to be analyzed. The molar ratio of antigen to total antibody in all of the immunomixtures was 4.3:1 (80 μg of CPA and 80 μg of total antibody dissolved in 0.2 mL of PBS, pH 7.4). Prior to their application to the sucrose gradient, the reaction mixtures were allowed to equilibrate for 24 h at 4 °C. Catalase (80 μg, M_r 245 000) and β-galactosidase (80 μg, M_r 595 000) were the molecular weight markers. The gradients were centrifuged for 15 h at 4 °C at 39 000 rpm using a Beckman ultracentrifuge equipped with an SW40 rotor. Fractions of 0.5 mL were collected from the top of the gradient using a Searle Densi-flow RII instrument, and the absorbance at 280 nm was constantly monitored by an ISCO VA-5 fluorescence absorbance monitor. The CPA enzymatic activity of each of the fractions was determined as described above.

Preparation and Characterization of CNBr Fragments. Cleavage of CPA with cyanogen bromide (CNBr) and separation of the fragments formed on Sephadex G-75 were performed according to Nomoto et al. (1969). The fragments were also separated by means of an immunoaffinity column prepared by Solomon et al. (1987). CPA and CPA fragments separated by gel filtration (F_1 and F_3) were adsorbed onto microtiter plates (10 μg/mL, 1000 μL), and direct and competitive ELISA experiments were performed.

Peptide Synthesis and Purification. Peptide P_1 [CPB (209–218), Asp-Tyr-Lys-Leu-Pro-Lys-Asn-Asn-Val-Glu] and peptide P_2 [CPA (209–218), Thr-Thr-Gln-Ser-Ile-Pro-Asp-Lys-Thr-Glu] were synthesized by the Merrifield solid-phase synthesis technique (Merrifield, 1964). Each of the peptides was purified by HPLC (Gilson, Model 303) on a Lichrosorb RP-18 column (12.5 × 0.4 cm; Merck, Darmstadt, West Germany). The column was run at a flow rate of 0.8 mL/min, collecting fractions of 1.6 mL. In a typical run, 4 mg of the crude peptide solution in 0.5 mL of PBS was applied to the column preequilibrated with 0.1% phosphoric acid in water (buffer A). After a 5-min wash with the same buffer, a linear gradient of 0–70% methanol in 0.1% phosphoric acid was applied over 55 min. Peaks were detected by the absorbance at 220 nm. A main peak of the purified peptide P_1 was eluted at fractions 16–19 corresponding to 32% methanol. Under the same conditions, peptide P_2 was eluted at fractions 14–17 (27% methanol). About 70% of the crude peptide was re-

covered. The peak fractions were pooled and concentrated about 20-fold by evaporation in vacuo in a Speedvac Savant centrifuge, and the resulting solution was neutralized to pH 7 by the addition of 1 N NaOH and diluted with PBS to a final concentration of 1 ng/mL. The purified peptide was analyzed for its amino acid composition (Heinrickson & Meredith, 1981).

Interaction of the Monoclonal Antibodies against CPA with Synthetic Peptides. The reaction of the monoclonal antibodies with the above purified synthetic peptides was determined by a competitive inhibition assay and dot immunoblotting. Each mAb (mAbs 100, 104, and 121, diluted 1:1000–1:18000 in PBS) was first incubated with each of the two peptides (50 μM in 1 mL of PBS for 1 h at 37 °C). Subsequently, 200 μL of these solutions was added to 96-well microtiter plates (Nunc, Roskilde, Denmark) precoated with CPA (1 μg/mL 0.05 M carbonate buffer, pH 9.6, 100 μL). After incubation for 1 h at 37 °C, the plates were washed with PBS, and peroxidase-labeled goat anti-mouse IgG was added. The color developed with *o*-phenylenediamine used as the substrate was proportional to the residual anti-CPA activity.

Dot Immunoblotting. Dot immunoblotting was performed according to Hawkes et al. (1982). Five micrograms (2.5 μL in PBS) of antigen solutions (P_1, P_2, CPA, and CPB) was applied on square pieces of nitrocellulose filter (0.45 μm) and dried at room temperature. After being blocked with 1 mL of cow milk for 30 min at room temperature, 400 μL of ascitic fluids of each of the mAbs (100, 104, and 121, mAbs 50 and 52 directed toward the active site of CPA, and an antibody which was unrelated to CPA, mAb DE-7) was added to each well and incubated for 2 h at 37 °C. After repeated washings of the filters with PBS, peroxidase-labeled goat anti-mouse IgG was added, and blue spots were developed with 4-chloro-1-naphthol in the positive wells.

Binding of CPA to Immobilized mAbs Preincubated with Synthetic Peptides. Sepharose–protein A beads (50 μL of a suspension containing 10 μg of protein A in 0.1 M phosphate buffer, pH 8.0) were incubated with ascitic fluids of each of the three mAbs (5 μg in 50 μL in the above buffer) for 1 h at room temperature. The beads were washed several times with buffer and incubated either with CPA or CPB or with the peptide fragment P_2 or P_1 (100 μL of 50 μM in PBS) for 1 h at room temperature. After repeated buffer washings, CPA (1 mg/mL, 0.5 mL) was added to the treated beads for 1 h, the beads were washed with buffer, and the enzymatic activity of bound CPA was determined as described previously (Solomon et al., 1987).

Results

Selection, Purification, and Characterization of Monoclonal Antibodies. The three mouse monoclonal antibodies to CPA with the highest titer (mAbs 100, 104, and 121) were selected, cloned, and used for the preparation of the corresponding ascitic fluids.

After isolation and purification using a protein A–Sepharose affinity column, they were examined for their effects on the enzymatic activity of CPA. None of them affected either the peptidase or the esterase activities of CPA.

Recognition of the Same or Overlapping Epitope(s) of CPA by the Three mAbs. The selectivity and saturation values of mAbs 100, 104, and 121 for different CPA epitopes were determined for each by an ELISA additivity assay (Ball et al., 1982; Djavadi-Ohaniance et al., 1984). The mAbs, either singly or in pairs, were allowed to saturate the adsorbed CPA, and the total amount of mAb adsorbed was determined by using the labeled second mAb. A double amount of the coating

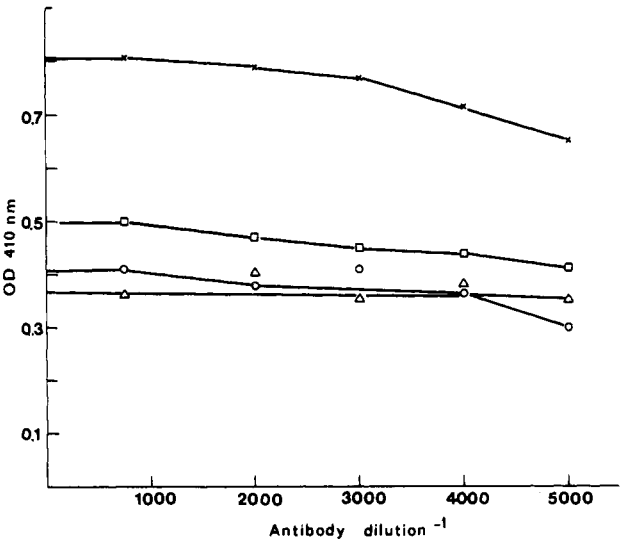

FIGURE 1: ELISA additivity test. Saturation curves of polystyrene-adsorbed CPA (100 μL per well containing 0.5–1.5 μg/mL) with mAb 100 by itself (○) or with the following pairs of mAbs: mAb 121 (△) and mAb 100–mAb 104 (□). The antibody dilutions given refer to the ascitic fluids containing the above mAbs. When pairs of mAbs were used, their ascitic fluids were diluted to the same extent and mixed 1:1 (v/v). The antibody dilutions given in the figure refer, in these cases, to the total antibody content. The OD_{410} values shown on the abscissa give the amount of the labeled secondary antibody bound. The upper curve (×) represents the binding of mAb 100 with the double amount of coated antigen.

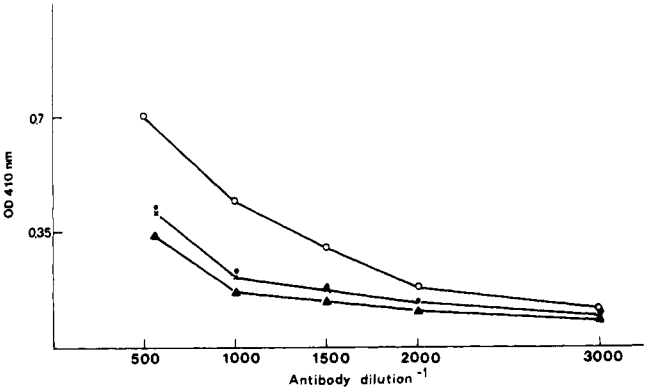

FIGURE 2: Binding of mAb 100–alkaline phosphatase conjugate at various initial concentrations (○) and its mixtures with unlabeled mAb 100 (●), mAb 104 (×), or mAb 121 (△) at the same molar concentration, with CPA adsorbed onto polystyrene ELISA plates. The abscissa gives the concentration of the labeled mAb in the various solutions employed, as expressed by the extent of dilution of the original solution containing 1 mg of labeled Ab per milliliter PBS, whereas the ordinate gives the amount of bound mAb 100–alkaline phosphatase as expressed by its enzymic activity.

antigen was used as a reference. As shown in Figure 1, in the presence of each pair of mAbs, the amount of bound mAb was similar to that bound when only a single mAb was employed, suggesting that the three mAbs either bind directly or bind close to the same epitopes.

The proximity of the epitopes was also demonstrated by an ELISA competition experiment using alkaline phosphatase labeled mAb 100 (Kearney, 1979). Competition between the labeled antibody (mAb 100–APase) and the other two nonlabeled antibodies (mAbs 104 and 121) for a given antigenic site of CPA was evaluated by means of ELISA experiments, in which the binding of mAb 100–APase to CPA adsorbed onto polystyrene plates was determined in the presence of mAb 104 or 121. Each of the unlabeled antibodies decreased the binding of the labeled antibody to nearly the same extent,

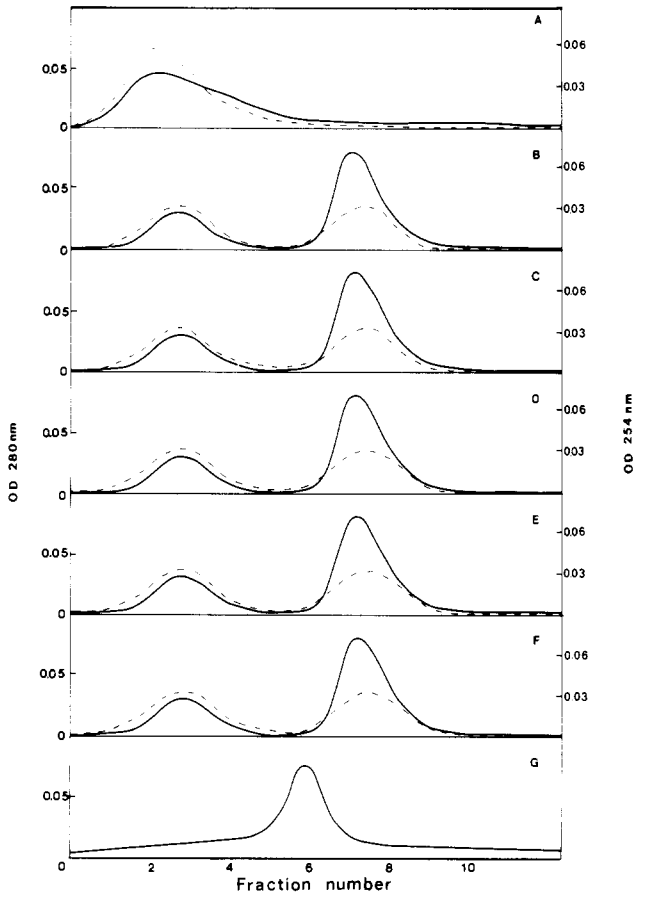

FIGURE 3: Summary of the sucrose gradient ultracentrifugation experiments carried out. A linear sucrose gradient ranging from 5 to 20% in PBS, pH 7.4, was prepared and samples of (A) CPA (16×10^{-5} M), (B) CPA + mAb 100, (C) CPA + mAb 121, (D) CPA + mAb 104, (E) CPA + (mAb 100 + mAb 104) (1:1), (F) CPA + (mAb 104 + mAb 121) (1:1), and (G) mAb 100. The molar ratio of antigen to total antibody was 4.3:1. Aliquots of 0.5 mL were collected from the top of the gradient with constant monitoring of the absorbance at 280 nm (—) and CPA enzymic activity (---).

reflecting competition of all three antibodies for the same or overlapping antigenic sites (see Figure 2).

In another set of experiments, the ELISA plates were coated with each of the unlabeled monoclonal antibodies followed first by incubation with CPA and then APase-labeled mAb 100. The resultant activities showed that the labeled monoclonal antibody did not bind.

In all experiments in which CPA was reacted with a single mAb, sucrose density sedimentation profiles of CPA mixtures with excesses of the three mAbs exhibit two peaks. The first corresponds to free CPA and the second to the CPA·mAb complex. The molar ratio of CPA/mAb was 2:1 in all three cases. Mixtures containing two monoclonal antibodies gave identical results (Figure 3), consistent with the conclusion that the three monoclonal antibodies bind to the same or overlapping antigenic regions on the CPA molecule.

Cross-Reactivity of Anti-CPA Antibodies with CPB. The tertiary structures of carboxypeptidases A and B are similar, and their amino acid sequences are 50% homologous (Titani et al., 1975). The cross-reactivity of CPB with anti-CPA mAbs was examined by binding of anti-CPA mAbs to CPB adsorbed on ELISA plates and by the binding of anti-CPA mAbs to CPA adsorbed in the presence of CPB. CPB did not react with anti-CPA mAbs in either case.

Recognition of the High Molecular Weight Fragment of CPA by mAb 100. CNBr splits CPA into four fragments which were isolated by gel filtration on G-75, resulting in three

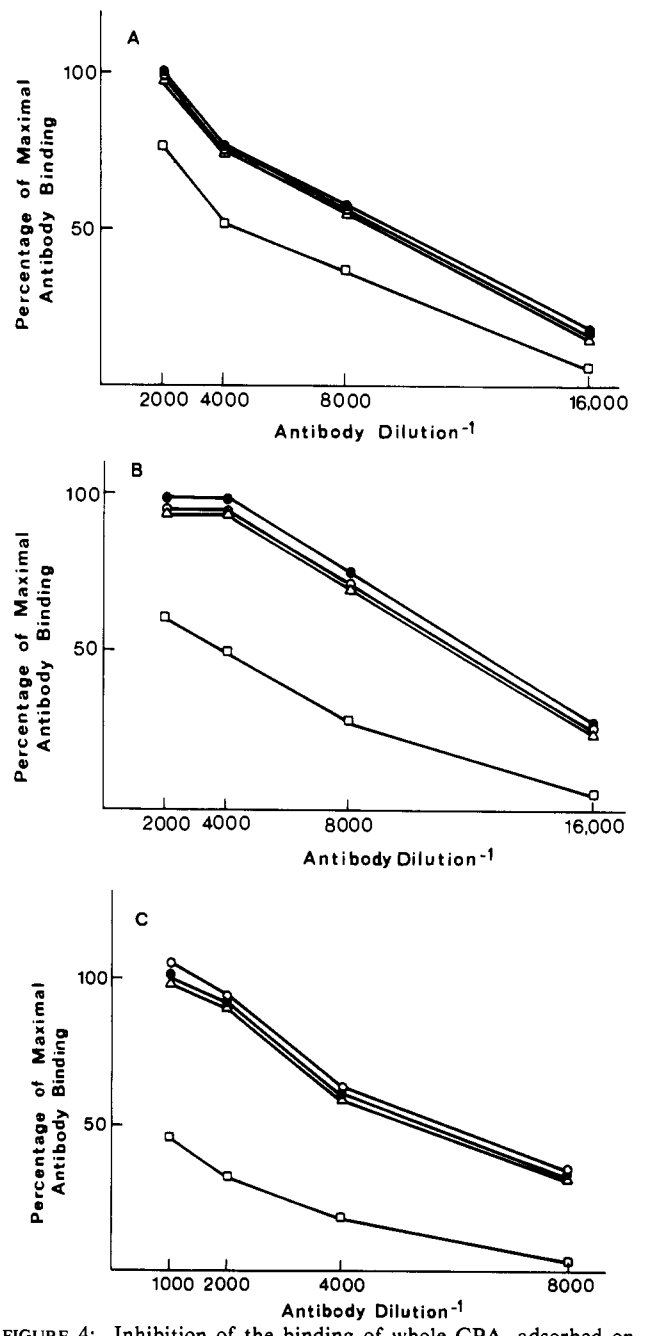

FIGURE 4: Inhibition of the binding of whole CPA, adsorbed on polystyrene ELISA plates, to mAb 104 (A), mAb 100 (B), and mAb 121 (C) by the peptides P_1 (△) and P_2 (□) and by CPB (○). Binding in the absence of inhibitors (●) is shown as a control (for details, see Materials and Methods).

major peaks, F_1, F_2, and F_3, in order of their elution. F_1 and F_2 actually represent the same fragment, F_1 constituting an aggregate of F_2 (Nomoto et al., 1969). Incubation of the CPA CNBr digest with mAb 100 immobilized on Eupergit C removes the F_1 and F_2 fragments from the eluate of the Sephadex G-75 column, but washing with 2 M propionic acid recovers them from the immunocomplex. mAb 100 recognized the antigenic determinant which is located in the F_1 and F_2 fragments of CPA, as results from competitive ELISA experiments.

Interaction of the Synthetic Peptides P_1 and P_2 with the Three mAbs. The three mAbs recognized the synthetic peptide P_2, which mimics the amino acid sequence 209–218 of CPA, and inhibited their binding to CPA (Figure 4). mAbs 100 and 121 bound the peptide more efficiently than did mAb 104, resulting in proportional degrees of inhibition.

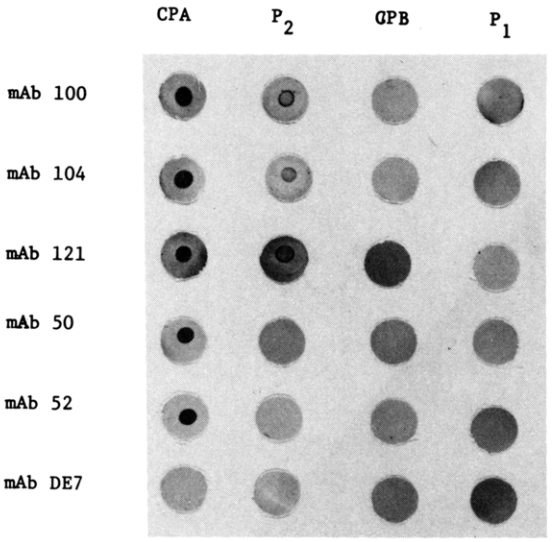

FIGURE 5: Dot immunoblotting assay for anti-CPA mAbs. Interaction between CPA, P_1, P_2, and CPB as antigens and mAbs 100, 104, and 121 was tested. As a control, mAbs 50 and 52 which bind to CPA at the active site and mAb DE7, unrelated to CPA, were used (for details, see Materials and Methods).

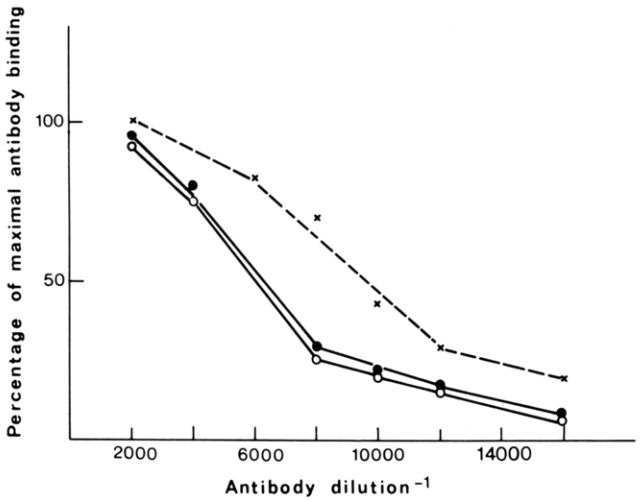

FIGURE 6: Binding profile of CPA (○), apoenzyme (×), and the reconstituted enzyme (●) to mAb 121 as determined by the ELISA procedure. The antibody dilutions given refer to the ascitic fluid employed which contained 0.8 mg of SPA-purified mAb 121/mL. The standard deviation in the experiments was 10%.

The three mAbs also specifically recognized P_2 in the immunoblotting assays (Figure 5). The interaction of P_2 with mAb 104 was weaker than that of mAbs 100 and 121, similar to the results obtained in the competitive assay described above. mAbs 50 and 52 directed toward the active site of CPA did not recognize P_2.

Preincubation of immobilized mAb 100 with P_2 inhibited the binding of CPA to this mAb. Preincubation of P_1 or CPB with this immobilized mAb did not affect CPA binding.

Detection of Conformational Changes in the Epitope Recognized by the Three mAbs. The apoenzyme prepared by dialysis of CPA against 1,10-phenanthroline (Auld & Vallee, 1970) is enzymatically inactive. Subsequent exposure to Zn^{2+} reconstitutes the holoenzyme and fully restores its activity. While mAbs 100 and 104 bind to the enzyme and apoenzyme in the same manner and to the same extent, mAb 121 differed significantly (Figure 6). Dilution of 1:10000 was required for its 50% binding to apo-CPA as compared with a dilution of 1:6500 for equivalent binding to native CPA. Addition of Zn^{2+} restored both enzymatic activity and concomitantly recovered the capacity of the native enzyme to bind to mAb 121.

Table I: Apparent Binding Constants Characterizing the Interaction of Monoclonal Antibodies with CPA Free in Solution or with CPA Immobilized by Adsorption onto ELISA Plates

mAb	ELISA[a] K_{app} (M^{-1})	solution K_{app} (M^{-1})
mAB 100	2.0×10^9	1.1×10^8
	2.8×10^9 [b]	
mAb 121	1.0×10^9	
	1.15×10^9 [b]	3.4×10^7
mAb 104	2.0×10^9	4.0×10^7
	4.0×10^9 [b]	

[a] Experimental conditions are as described in the text. [b] CPA was pretreated with urea prior to its adsorption on the ELISA plates (see Materials and Methods). Each figure in the table represents the mean of data obtained from five experiments with 10% standard deviation.

Binding constants for the three monoclonal antibodies to CPA were determined for two different physical states of the enzyme, immobilized and when free in solution. Each of the three monoclonal antibodies bound to CPA adsorbed to polystyrene plates with a binding constant ranging from 1.0×10^9 to 2.0×10^9 M^{-1} (Table I). Pretreatment of CPA with 8 M urea prior to coating on the ELISA plates changed the binding characteristics of these monoclonal antibodies, increasing its interaction with mAbs 100 and 104.

The apparent binding constants of the enzyme when free in solution were deduced from Scatchard plots representing the experimental data obtained with the procedure given under Materials and Methods. Those for the binding of CPA to the antibodies in solution were lower by 1 or 2 orders of magnitude than the ones obtained for the corresponding immobilized enzyme–antibody systems (Table I).

Discussion

We have previously described the preparation and purification of several monoclonal antibodies to CPA (Solomon et al., 1984a), a well-characterized zinc exopeptidase that exhibits both peptidase and esterase activities. Some of them primarily inhibit peptidase activity and others the esterase activity while yet additional ones affect both. Finally, some of their antibodies bind to the enzyme but do not noticeably affect any of its catalytic properties. Most of the antibody–enzyme complexes are characterized by binding constants of the order of 10^6 M^{-1} (Solomon et al., 1984a).

It requires high binding constants to qualify mAbs as suitable probes to detect subtle conformational alterations of CPA. The three mAbs selected for the present study meet this requirement, as their apparent binding constants are of the order of 10^9 M^{-1}. They bind to the same or to overlapping regions in the primary sequence in the vicinity of amino acid residues 209–218.

The monoclonal antibodies mAb 100 and mAb 104, derived from the same fusion experiment, belong to subclass IgG1 and IgG2a, respectively. The monoclonal antibody mAb 121 of IgG1 originated from a different fusion. The binding characteristics of these three mAbs to native CPA either in solution or when adsorbed onto an ELISA plate confirm that the identities of the three mAbs differ, and they recognize the same or partially overlapping antigenic sites. The following findings support this conclusion: (a) A combination of any two of the three mAbs is not additive in the standard ELISA assay (in which CPA is adsorbed onto the polystyrene plate) (Figure 1). (b) Sucrose gradient ultracentrifugation of the immunocomplex of either one or any two of these mAbs with CPA results in the same molecular weight (Figure 3). (c) In ELISA experiments in which CPA is adsorbed onto polystyrene, mAb 100 labeled with alkaline phosphatase competes with any of

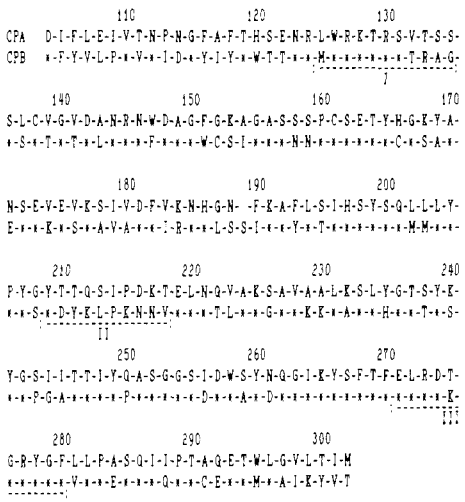

FIGURE 7: Amino acid sequences, represented as one-letter codes, of the F_1 region of CPA and the corresponding region in CPB. The three peaks of high hydrophilicity are underlined (see Figure 8).

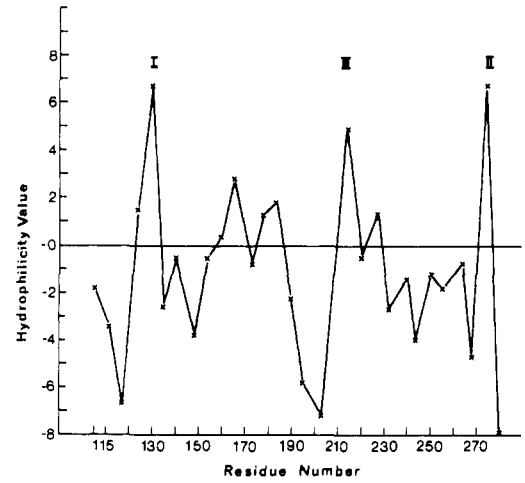

FIGURE 8: Hydrophilicity profile of the F_1 fraction plotted against residue numbers. Values were averaged over groups of six residues. The location of regions with high hydrophilicity are indicated as I, II, and III.

the three unlabeled mAbs (Figure 2). (d) alkaline phosphatase activity could not be detected on ELISA polystyrene plates after any one of the unlabeled mAbs had been adsorbed, interacted with CPA, and then treated with mAb 100–alkaline phosphatase conjugates.

The antigenic site under consideration and the active site of the enzyme do not coincide, since enzymatic activity of CPA is preserved on complex formation with the mAbs. This antigenic region encompasses fragment F_1 (amino acid residues 104–302) of CPA obtained by cyanogen bromide cleavage. This fragment was adsorbed to an affinity column of mAb 100 conjugated to Eupergit C and was also recognized by the three mAbs in direct and indirect ELISA assays.

The antigenic site in question is a sequential one as suggested by the fact that binding constants to denatured antigen–mAbs complexes are higher than those of the complex with the native antigen. These three mAbs do not recognize CPB. Comparison of the structure of CPA with that of CPB sheds some light on the location of the region of CPA which recognizes the mAb (Figure 7).

In order to get further information as to the location of the antigenic determinant in question, application of some of the predictive criteria for the localization of antigenic sites in proteins was attempted. Hydrophilicity, accessibility, and mobility have been thought to be the three best parameters to predict antigenicity (Parker et al., 1986), and several computer programs have been developed on the basis of a hydrophilicity scale (Hopp & Woods, 1981; Kyte & Doolittle, 1982). These programs successively evaluate the sequence of a protein over an interval of five to nine amino acids proceeding from the amino to the carboxy terminus of the polypeptide. Because of lack of information in our hands of the accessibility and the mobility characteristics of CPA, only the hydrophilicity profile of CPA was employed for further consideration. Figure 8 shows the hydrophilicity profile of F_1 according to the procedure of Kyte and Doolittle (1982). On the basis of their criteria, there are three regions of the F_1 fragment in which the antigenic site of the above three mAbs would most likely be located: (I) 125–135; (II) 209–218; and (III) 271–281. The first peak reflects residues 125–135 in a region near the active site of the enzyme. Since the mAb investigated does not inhibit peptidase or esterase activities of CPA at all, this region does not seem to be the antigenic determinant in question. Peak III located between residues 271 and 281 of CPA resembles the primary structure of CPB, suggesting that this region is not the putative binding site either, since the three mAbs for CPA do not cross-react with CPB. The residues between 209 and 218 constitute the only sequence which would seem to correspond to a high hydrophilicity peak, not homologous to CPB, remote from the active site of the enzyme and yet at the surface of CPA. Hence, all three mAbs might recognize this epitope. Therefore, peptides corresponding to the sequence 209–218 in both CPA and CPB were synthesized and tested for their capacity to inhibit the formation of the antibody–CPA complex. There was competition when each of the three mAbs incubated with a large excess of peptide P_2 (209–218 CPA), while there was none in the presence of peptide P_1 (209–218 CPB) (Figure 4). Direct binding studies by dot immunoblotting showed that the three mAbs recognize P_2 but not P_1 (Figure 5). Other anti-CPA mAbs directed toward a different region of the molecule inhibiting CPA activity did not recognize the P_2 peptide.

Thus, all of the experimental data suggest that peptide P_2 (CPA 209–218) mimics the antigenic determinant of CPA with respect to the above mAbs and in accord with prediction. The conformational characteristics of this epitope were examined by comparing binding of the mAbs to native CPA in solution with that to CPA adsorbed onto polystyrene plates. When CPA is adsorbed onto ELISA polystyrene plates, the binding constants of all three mAbs (1.0×10^9 to 2.0×10^9 M^{-1}) are considerably higher than when the native enzyme is in solution (4.0×10^7 to 1.13×10^8 M^{-1}) (Table I). This suggests that CPA, when adsorbed to polystyrene, undergoes conformational changes such that epitope (209–218 CPA) is exposed to the surface solvent. Well-defined mAbs interact differently with their corresponding protein antigen when in solution than when adsorbed onto polystyrene plates (Frackelton & Rotman, 1980; Friguet et al., 1984; Mierendorf & Dimond, 1983). In this regard, one of the mAbs against human lactic acid dehydrogenase isoenzymes (HLDH5) is highly specific toward the antigen when adsorbed onto a polystyrene plate, while when in solution it does not recognize the isoenzyme at all (Hollander & Katchalski-Katzir, 1986).

Table I shows that the apparent binding constant for the denatured CPA·mAb 121 complex closely resembles that observed for the adsorbed, untreated enzyme but that of the complex of the denatured enzyme with mAb 104 differs markedly. All of the values were somewhat higher than those for binding to the native untreated enzyme, suggesting that urea treatment of CPA prior to its adsorption apparently

exposes additional mAb-recognizing groups; the three mAbs recognize the denatured enzyme differently.

These studies also indicate that the conformation of the apoenzyme differs from that of the native enzyme to an extent which is recognized by mAb 121 and only slightly by mAbs 100 and 104 (Figure 6). The conformational changes which occur upon Zn removal are fully reversible, as both enzymatic activity and immunological characteristics of the native enzyme are fully restored upon reincorporation of Zn. In crystallographic studies (Rees et al., 1986), data collected at high resolution (1.5–1.8 Å) could not establish if the metal binding site is created independently by the protein or if metal–protein interactions are required to induce its binding site. The structures of apoenzyme and native enzyme appear to be similar within the limitations imposed by this method. The highly specific monoclonal antibodies suggest that changes occur even remote from the active site. It should be mentioned, moreover, that the various isoenzymes of CPA respond to metal removal or changes in pH and temperature in a manner so different that subtle conformational alterations may lead to major alterations in the functional and physical states of the enzyme.

While the binding of the monoclonal antibody to CPA does not alter esterase or peptidase activities, they are sensitive to conformational changes in different functional states of the enzyme, likely due to their high and specific binding affinities to a particular peptide fragment of the enzyme. Even slight changes in the conformation of this peptide fragment may lead to an amplified response to antibody binding. In this regard, antibody probes may be useful to determine whether or not conformational changes occur in an enzyme at distances remote from the active site during the different elementary steps of catalysis.

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