The Enzymic Behavior of Carboxypeptidase-A in the Solid State*

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ABSTRACT: Crystals of carboxypeptidase-A, cross-linked by treatment with glutaraldehyde to give an insoluble network [Quiocho, F. A., and F. M. Richards (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 833], have been assayed by a flow-through column procedure with carbobenzoxyglycyl-L-phenylalanine as substrate under a variety of conditions. At pH 7.5 and low ionic strength the maximum specific activity of very small native crystals was 300 times less, and for the amorphous enzyme 70 times less, than that of the soluble form. The cross-linking reaction decreased the activity by about a factor of 4 for the amorphous material and 2 for the crystals. Analyses of acid hydrolysates of cross-linked material showed a loss only of lysine residues. The great stability of the cross-linked crystals was shown by a single column used for many assays over a period of 3 months with no change in specific activity.

The concentrations at half-maximum activity for all samples, including crystals of varying sizes, amorphous and soluble forms, were in the range 0.01–0.02 M. The maximum specific activities for the samples of the smallest crystals were independent of size, indicating an absence of diffusion limitation. Substrate inhibition is qualitatively different in crystals from that in solution but does not require a different mechanism. Terms in $(S)^3$ in the rate equation indicate more than one modifier binding site in addition to the catalytic site. Such interactions may cause inhibition or activation depending on the substrate. The inhibition constant for β-phenylpropionic acid was about 10^{-4} M for the amorphous and soluble forms of the enzyme, but was increased to about 10^{-2} M for the crystals. The chelating agent, 1,10-phenanthroline, inactivates the amorphous material presumably by removing the atom of zinc as in solution. The activity is fully recovered upon readdition of the zinc. In columns of crystals at either low ionic strength or in 1.5 M NaCl, the activity is eliminated in the presence of 1,10-phenanthroline, and recovered merely by washing the crystals with deionized water. The zinc is not removed, and the inhibition is apparently caused by ternary complex formation. For both the amorphous material and the crystals the rate of activity loss on treatment with the complexing agent is slowed in the presence of substrate. Many of the properties of carboxypeptidase-A that are seen in solution are also observed in the crystals, but many changes in the detailed behavior of the enzyme occur in passing from solution to the solid state.

Studies on the chemistry of proteins in the crystalline state have gained impetus in recent years from the high resolution structures now known for myoglobin (Kendrew, 1963) and lysozyme (Blake *et al.*, 1965) and from the many other such structures on the horizon (Dickerson, 1964). For those proteins which happen to be enzymes and which have demonstrable activity toward low molecular weight substrates, catalytic behavior in the crystalline state can be investigated. In principle, the structures of the enzyme and its complexes with substrates, products, inhibitors, and activators may be determined by diffraction techniques. The significance of such structures will depend on knowledge of the chemical behavior of the protein in the environment provided by its lattice site in the crystal. The property of interest may or may not be identical with that for the same enzyme in dilute aqueous solution.

Ribonuclease-S (Doscher and Richards, 1963), ribonuclease-A (Bello and Nowoswiat, 1965), and chymotrypsin (Sigler and Skinner, 1963) have been reported to be catalytically active in the crystalline state. Since the liquid-filled channels in the crystal lattices are small, restricted diffusion for substrates of even moderate size may be marked. For crystals in the 100-μ range, or larger, and for reactions with half-times in solution of a few minutes or less, diffusion limitation of the rate may be expected to be severe. As a result, the characterization of the kinetic parameters applicable to crystals of the size normally used for diffraction studies has been difficult. However, by observing the effect of crystal size and by studying the interaction of inhibitors or activators which do not "turn over," some of these difficulties can be circumvented.

This paper reports kinetic studies on carboxypeptidase-A in the solid state. The enzyme was chosen initially

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because of its insolubility in dilute salt solutions. However, even in this case, solubilization of the usual crystalline material by many of the solvents of interest became a problem. This difficulty was overcome by intermolecularly cross-linking the protein molecules in single crystals to give completely insoluble three-dimensional nets. The majority of the experiments reported in this paper have been performed on crystals treated with various bifunctional reagents, particularly glutaraldehyde. The catalytic activity of the cross-linked derivative of the native protein toward the hydrolysis of a dipeptide substrate has been investigated, and, where possible, comparisons have been made with noncross-linked crystals. A brief report of the early phase of this work has been published (Quiocho and Richards, 1964).

Materials

Five-times crystallized carboxypeptidase-A, prepared by the method of Anson (1937) was obtained as a suspension, with toluene as preservative, from Calbiochem, Inc., lot no. 21735; chromatographically pure carbobenzoxyglycyl-L-phenylalanine from Mann Research Laboratory, lot no. F3916 and L2156; β-phenylpropionic acid from Eastman Organic Chemicals; reagent grade 1,10-phenanthroline monohydrate from Matheson Coleman and Bell; glutaraldehyde as a 25% aqueous solution from Aldrich Chemical Co. The concentrations of stock solutions of the enzyme were estimated from absorbancy at 278 mμ using an extinction coefficient of 6.4×10^4 l./mole cm (Simpson *et al.*, 1963).

Methods

Steady-State Column Assay Procedure. A piece of glass capillary tubing about 1.7-mm i.d. was tapered and fitted with a small plug of cotton. The column was filled with water and the crystals, or other preparation of the enzyme, inserted with a pipet, and allowed to settle. The final height of the packed crystals was usually 3–5 mm. A polyethylene tube (PE 60, Clay Adams Inc., N. Y.) was inserted as a press fit into the top of the column and connected to an "Agla" micrometer syringe at the other end. The system was completely liquid filled. Air bubbles were carefully eliminated. The syringe was driven by a 1-rpm synchronous motor and produced a flow rate through the column of 10 μl/min. The syringe was filled with the appropriate substrate solution or wash liquid.

In the peptidase assay with carbobenzoxyglycyl-L-phenylalanine as substrate, each drop of the column effluent was collected in a separate 16 × 180 mm test tube and the ninhydrin color was developed immediately according to the procedure of Moore and Stein (1948). When the absorbance at 570 mμ reached a constant value, indicating a steady state within the column, the drop collection was discontinued. Generally 8–10 drops were required. Activity was defined as the plateau absorbance value at 570 mμ corrected for the reagent blank and normalized to the volume equivalent to a drop time of 1 min. The actual drop time varied, of course, with the composition of the liquid. On occasion three additional drops were collected and allowed to stand for various lengths of time before addition of the ninhydrin solution in order to test for the presence of soluble enzyme.

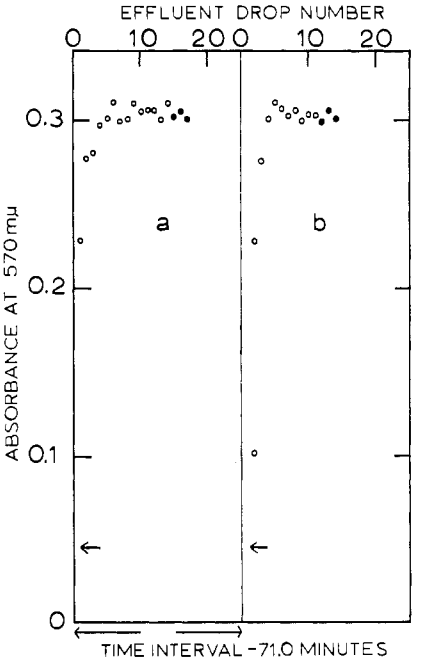

FIGURE 1: Peptidase activity of large native carboxypeptidase-A crystals determined by the column assay procedure. The assay solution was 0.02 M carbobenzoxyglycylphenylalanine in 0.02 M sodium veronal, pH 7.5. The liquid flow was 10 μl/min and the drop time 2.88 min. The ninhydrin color was developed in each drop immediately after collection except for the last 3 drops (filled circles) in each run which were kept for 10, 20, and 30 min, respectively, before addition of the ninhydrin reagent. Part a shows the initial assay on this particular column of crystals. Part b is a repeat assay made on the same column eight days later. The plateau absorbance corresponds to 17.5% hydrolysis of the substrate. (Complete hydrolysis of the substrate in a single drop gave an absorbance at 570 mμ of 1.44.) The arrow on the left ordinate shows the absorbance of a single drop of substrate solution in the absence of enzyme.

Unless otherwise indicated the standard peptidase assay was carried out in 0.02 M sodium veronal buffer at pH 7.5 and 0.02 M carbobenzoxyglycylphenylalanine. A typical assay is shown in Figure 1. The temperature was not controlled, but during an assay series room temperature usually varied less than 2° and was about 25°.

Interconversion of the numbers obtained from column assays with those obtained from measurements in solution or on suspensions of solid enzyme

are occasionally required. Specific activity in the column system is expressed as ΔA_{570}/(min/mg of enzyme), and is equivalent to the total amount of substrate converted to product per unit time per unit weight of enzyme for a substrate solution of specified composition. In the usual solution assays aliquots, α (milliliters), are removed from a known volume of assay solution, V (milliliters), containing a total weight of enzyme, E (milligrams). The ninhydrin color is developed with aliquots taken at various intervals after the initiation of the reaction. The slope of the line of ninhydrin color *vs.* time gives ΔA_{570}/min. This number is multiplied by $V/\alpha E$ to give a specific activity equivalent to that in the column assay with the same substrate solution.

The column assay was also used with amorphous enzyme preparations. A layer of Whatman ashless cellulose powder was layered on top of the cotton plug in the glass column. The amorphous enzyme in the form of a slurry with more cellulose powder was then poured as in the preparation of an ordinary chromatographic column. The amount of the slurry finally used was determined by a few preliminary assays in order to get a convenient per cent conversion of the substrate solution.

Preparation and Optical Measurement of Crystals. Crystals of carboxypeptidase-A were grown from solutions of the enzyme in 1 M sodium chloride by slow dialysis in collodion sacs at 2° against decreasing salt concentrations. The solutions were buffered at pH 7.5 with 0.02 M sodium veronal and HCl. The crystals in any given sac tended to be reasonably uniform in size and shape. However, the size varied markedly between different tubes.

Crystals were removed from the sacs and gently washed twice with water in a 6 × 50 mm test tube. They were immediately cross-linked with glutaraldehyde (see below) in order to avoid chipping, cracking, or solubilization upon subsequent treatment, and were further fractionated by sedimentation to obtain a more uniform size. Water was added to the tube from a micropipet so as to disperse clumps of crystals. The test tube was then filled completely, covered with parafilm, and inverted until all the crystals were resting on the parafilm. The tube was rapidly reinverted permitting the crystals to fall freely to the bottom of the tube. The settling time was chosen for the size of crystals required. The solution containing the slower sedimenting crystals was immediately removed with a wide orifice pipet. By repeating several times, a more homogeneous size distribution was obtained. These sized batches were then mounted in separate columns for assay. Five batches of cystals differing in size were obtained.

The weight and approximate average dimensions of a given batch of crystals were determined as follows. (All operations were done in an electron microscope laboratory where the air was relatively dust free.) The column of crystals was washed with 1.5 ml of clean distilled water using the flow system. The crystals were transferred quantitatively to a tared Corning cover glass (24 × 40 mm) by forcing distilled water through the bottom end of the column with the micrometer syringe. The crystals were dried to constant weight over anhydrous $CaCl_2$ in a dessicator at room temperature.

Calibrated photomicrographs of the dried crystals permitted direct measurement of two dimensions. The specimens were shadowed with chromium, as for electron microscope samples, to obtain an estimate of the third dimension, the crystal thickness. A Kinney SC-3 vacuum evaporator was used. A "point" source of chromium was obtained by placing a 0.15-mm thick stainless steel mask with a 1.5-mm hole in front of the tungsten basket holding the chromium chips. Because of the substantial size of the total sample the shadowing angle varied in different parts of the sample. The angles were calculated for different parts of the coverslip from the positioning measurements. These angles were checked by placing small pieces of cover glass, 0.14 mm thick, on various parts of each sample and using the shadows cast by these standards for further calibration. The thickness of the glass chips estimated from the shadowing data was 0.146 ± 0.006 mm. Other parts of the same coverslip measured with a micrometer caliper gave 0.14 mm. Enlarged photographs of the entire field of each sample were prepared. The total number of crystals was obtained by direct count. The three dimensions of a number of crystals selected from throughout the field were measured and averaged for each sample.

Amino Acid Analysis. Analyses were carried out by the method of Spackman *et al.* (1959) using 50-cm columns. All samples were hydrolyzed in 6 N hydrochloric acid at 110° in sealed evacuated ampules for 24 hr.

Intermolecular Cross-Linking. Unless otherwise specified, crystals of carboxypeptidase-A were cross-linked by suspension in a 1% aqueous solution of glutaraldehyde buffered at pH 7.5 with 0.02 M sodium veronal at room temperature for a period of about 1 hr. The excess glutaraldehyde was removed by washing the crystals repeatedly with water. It was also possible to cross-link the crystals in the flow system used in the steady-state assay. Native crystals, which were carefully mounted in a column, were cross-linked by flowing the glutaraldehyde solution with the motor-driven micrometer syringe. In this way it was possible to assay a crystal batch before and after cross-linking without removing the crystals from the column or disturbing their packing.

Amorphous cross-linked carboxypeptidase-A was prepared as follows. A 75-μl aliquot of a solution containing about 0.13 mg of protein was treated with an equal volume of 4% glutaraldehyde solution buffered with 0.02 M sodium veronal at pH 7.5. The reaction was allowed to proceed for 1 hr during which time a precipitate formed. The precipitate was recovered by centrifugation and the excess glutaraldehyde was removed by washing. When used in the form of a column, this material was mixed with cellulose powder in order to allow adequate flow through the packed sample.

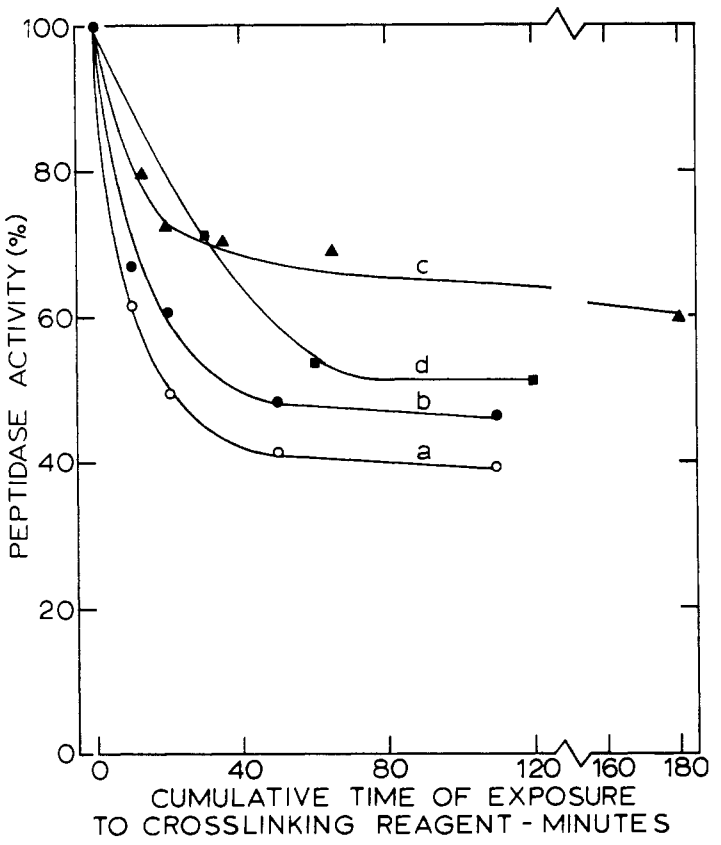

FIGURE 2: Effect of the cross-linking reaction on the activity of carboxypeptidase-A crystals. The data for four separate columns of crystals are shown. The initial activity of each column in the standard assay was set at 100%. Solution containing the cross-linking reagent was then flowed over the particular column for the time indicated by the first point on the curve. Here the column was washed with water for 15 min, used in the standard assay, washed with water again for 15 min, then returned to the cross-linking solution for the next time interval. The results of the consecutive assays, given as per cent, are shown on the ordinate. The cross-linking solutions in all cases contained 0.02 M sodium veronal buffer, pH 7.5. Other components were as follows: curve a, —○—, 1% glutaraldehyde; curve b, —●—, 1% glutaraldehyde and 0.01 M β-phenylpropionate; curve c, —▲—, 0.1% glutaraldehyde; curve d, —■—, 0.007 M difluorodinitrobenzene and 17% ethanol. (Note that in curve b the column was washed with buffer solution containing 0.01 M β-phenylpropionate for 30 min before the cross-linking reaction was started.)

Results

Cross-Linking Reaction. All experiments were done in 0.02 M sodium veronal buffer at pH 7.5 containing the appropriate concentration of glutaraldehyde and any other substances as indicated. The native crystals were either gently suspended in these solutions or packed in a column and the solutions were passed over them in the flow assembly. The treatment in either case was carried out at room temperature.

The effect of the cross-linking reaction on the activity of crystals in the standard peptidase assay is shown in Figure 2. In all the measurements so far made the final measured activity has been between 30 and 70% of the starting value for the particular crystal batch. The course of the reaction was detectably but not dramatically effected by the presence of the competitive inhibitor, β-phenylpropionate (Figure 1, curves a and b). For subsequent measurements, unless otherwise indicated, crystals were cross-linked in 1% glutaraldehyde for 1 hr.

For one column of cross-linked crystals esterase activity was tested using hippuryl-DL-β-phenyllactate, 0.001 and 0.005 M, as substrate in 0.2 N NaCl, 0.005 M Tris buffer, pH 7.5. Hydrolysis was detected by the increase in absorbance at 254 mμ. The activity found was clearly due to the solid and not to soluble enzyme since the A_{254} of the column effluent did not change with time. No detailed studies with this substrate have yet been made.

The increase in mechanical strength of the crystals after cross-linking was noted in the earlier paper (Quiocho and Richards, 1964). Although it is possible by using high concentrations of reagent to produce crystals which effectively cannot be broken, more gentle reaction conditions yield material which still can be pulverized. To check that the cross-linking reaction did involve the whole crystal lattice, aliquots of some

TABLE I: Amino Acid Composition of Crystals of Carboxypeptidase-A after Treatment with Glutaraldehyde.[e]

Amino Acid	Number of Residues/Molecule			
	Native Enzyme		Cross-Linked Crystals	
	Lit. Values[a]	This Sample[b]	Glutaraldehyde (1%, 1 hr[c])	Glutaraldehyde (6%, 1 hr[d])
Lysine	15.01	15.0 ± 0.8	6.1 ± 0.2	4.1 ± 0.4
Histidine	7.71	8.0 ± 0.4	6.8 ± 0.2	6.9 ± 0.4
Arginine	9.88	10.3 ± 0.5	11.0 ± 0.4	10.7 ± 0.4
Aspartic acid	26.50	27.9 ± 0.4	27.9 ± 0.8	28.7 ± 0.8
Threonine	23.40	23.1 ± 0.7	23.5 ± 0.2	23.0 ± 1.1
Serine	30.00	28.3 ± 0.7	27.8 ± 0.2	27.0 ± 0.4
Glutamic acid	24.93	24.2 ± 0.3	25.7 ± 0.2	25.6 ± 0.5
Proline	9.83	10.9 ± 0.4	11.0 ± 0.6	11.2 ± 0.1
Glycine	22.45	22.9 ± 0.8	23.5 ± 0.3	22.3 ± 1.4
Alanine	19.20	19.8 ± 0.3	20.1 ± 0.1	20.3 ± 0.1
½-Cysteine	2	0.8 ± 0.3	1.4 ± 0.1	1.2 ± 0.4
Valine	15.60	16.3 ± 0.7	15.5 ± 0.2	16.1 ± 0.5
Methionine	2.67	3.3 ± 0.3	2.5 ± 0.3	3.2 ± 0.4
Isoleucine	19.80	18.5 ± 0.4	17.7 ± 0.2	18.2 ± 0.4
Leucine	22.90	22.9 ± 0.5	23.1 ± 0.7	22.8 ± 0.1
Tyrosine	18.80	17.0 ± 0.5	17.5 ± 0.4	17.1 ± 0.4
Phenylalanine	14.56	15.3 ± 0.6	14.9 ± 0.9	14.7 ± 0.2

[a] Corrected values were taken from Bargetzi *et al.* (1963). [b] Standard deviation was calculated from a total of five analyses on three separate hydrolysates. [c] Total of three analyses of two separate hydrolysates. [d] Two analyses on a single hydrolysate. [e] No analyses for tryptophan were carried out. Values are not corrected for losses on hydrolysis.

preparations were removed at various times during the reaction, washed, crushed, extracted with 1 M NaCl, and the clear supernatant fluid was assayed for enzymic activity. With 0.1% glutaraldehyde as the reagent, activity was found in the early extracts; but after 30–40 min there was no soluble enzyme with the particular size of crystals used. The crystals gave activity comparable to that shown in Figure 2, curve c. It is clear that the entire crystal is affected by the reaction and that a true three-dimensional net is formed.

The stability of these cross-linked crystals is notable. One particular column was used intermittently over a period of 3 months. Some 67 different assays were carried out under a variety of conditions. From time to time the standard assay was performed as a control. In nine such standard assays during the 3-month period there was no trend in the measured activity. The value of ΔA_{570}/min was 0.066 with a standard deviation over the nine runs of ±0.002. When not in use the column was kept immersed in water at room temperature.

Representative amino acid analyses of crystals treated with glutaraldehyde are shown in Table I. Substantial and consistent changes in composition were observed only for lysine. The variations for all other amino acids were probably within the error of the analyses. Cross-linked crystals which were exposed to solutions of sodium borohydride before acid hydrolysis gave analyses indistinguishable from those of the untreated materials. Again lysine was the only amino acid affected. It is clear that, if the cross-linking reaction involved residues other than lysine, the conditions of hydrolysis regenerate the amino acids from such products. It is also clear that lysine is not regenerated from the product of the aldehyde reaction. The lysine contents of preparations cross-linked under various conditions are shown in Table II. It should be noted that no determinations of tryptophan were carried out and thus no statement can be made as to whether or not this particular amino acid is affected by the cross-linking reaction.

Cordes and Jencks (1962) have shown that Schiff bases involving aniline and aryl aldehydes are rapidly attacked by semicarbazide to yield a semicarbazone and the free amine. The stability constants for the semicarbazones are many orders of magnitude greater than those for the Schiff bases. There is no equivalent information available for aliphatic aldehydes and amines, but operating by analogy, attempts were made to reverse the cross-linking reaction with semicarbazide. Treatment of cross-linked crystals with semicarbazide, even in concentrations as high as 4 M, failed to produce any evidence of solubilization either by inspection or by assay of the liquid phase. As a result, it is assumed that the reaction of glutaraldehyde and the ε-amino group of lysine has proceeded beyond the stage of

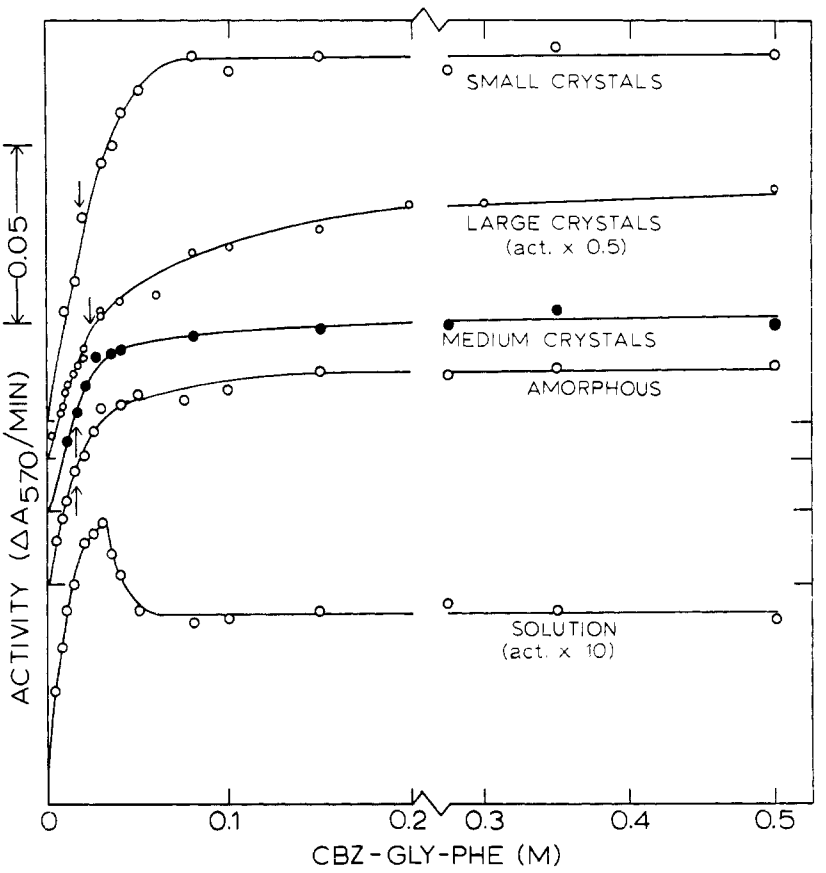

FIGURE 3. Plots of activity *vs.* substrate concentration for the hydrolysis of carbobenzoxyglycyl-L-phenylalanine by carboxypeptidase-A in various forms. The stock substrate solution was made up in 0.02 M sodium veronal, pH 7.5. The ionic strength was maintained at 1.5 with the inclusion of the appropriate concentration of NaCl taking into account the contribution of the substrate. The crystals and amorphous enzyme were assayed by the column technique. Further characterization of the columns of crystals is given in Table III. The column of amorphous material contained 0.045 mg of enzyme. The numbers used as ordinates are the measured ninhydrin values for 10 μl of the column effluent (1 min) corrected for the reagent blank, except for the curve labeled "large crystals" where the observed values have been divided by 2. For clarity the origins have been displaced on the ordinate. The arrows indicate the substrate concentration at which the rate is one-half of the maximal activity. In the assay for the dissolved enzyme, aliquots of 100 μl were taken from 1 ml of assay solution containing 0.5 μg of enzyme. The ninhydrin color of each aliquot was developed immediately. Activity was taken as the slope of the plot of A_{570} *vs.* time. The numbers so obtained have been multiplied by 10 and plotted on the same scale as the column values. If it had been possible to do a "column assay" on the soluble enzyme, the curve shown would correspond to a column containing 0.5 μg of enzyme. The numerical conversion is discussed in the methods section.

simple Schiff base formation.

Other bifunctional reagents were tested for their ability to intermolecularly cross-link carboxypeptidase in the solid state. Failure to dissolve in 2 N NaCl was taken as evidence that cross-linking had, in fact, occurred. Treatment with 0.002–0.007 M 1,3-difluoro-4,6-dinitrobenzene in 10% ethanol gave yellow insoluble crystals. The time course of the activity change during this reaction is shown in Figure 2, curve d. Biphenyl-4,4′-bisdiazonium dichloride was also used successfully. However, the aryl dialdehyde, terephthaldehyde, produced no detectable cross-linking when used as a saturated solution in 50% aqueous ethanol. The effects of the reagents other than glutaraldehyde have not yet been investigated in any detail.

Enzymic Behavior of Carboxypeptidase in the Solid State. The effect of varying the concentration of the peptide substrate on the activity of carboxypeptidase in various forms is shown in Figure 3. In an effort to outline the severity of the problem of diffusion limitation, activity measurements were made on five batches of crystals with different average sizes. Crystals having as uniform a size as possible within each batch were selected and labeled large, medium, small, 4, and 5. The dry weight of each batch was determined after the assays. The corresponding activities at two levels of substrate concentration were taken from the data in Figure 3. The thickness of the crystals was determined

TABLE II: The Lysine Content of Cross-Linked Crystals of Carboxypeptidase-A.[c]

Glutaraldehyde Concn (%)	Reaction Time	Residues of Lysine/ Enzyme Molecule
0	0	15.0
0.1[a]	10 min	9.2
0.1	1 hr	4.6
1	10 min	8.4
1	1 hr	6.1
1[b]	1 hr	4.1
6	1 hr	4.1
6	3 hr	1.6

[a] Insolubilization of crystals incomplete. [b] Crystals were treated with 0.05 M sodium borohydride at pH 9.5 for about 2.5 hr after the cross-linking reaction and before acid hydrolysis. [c] Cross-linking reaction carried out in 0.02 M sodium veronal buffer, pH 7.5, at room temperature.

by the shadowing technique. The total number of crystals and the other two dimensions were taken directly from the photomicrographs. The approximate surface area was estimated as follows. Since the shapes (*i.e.*, axial ratios) of the crystals in all the batches were very similar, then for a given crystal $a^{1/2} \sim w$, where a and w are the surface area and weight of the crystal, respectively. For a batch of n uniform crystals the total area is na and the total weight nw. Thus the total area of a crystal batch is proportional to $n^{1/2}W^{1/2}$, where W is the total batch weight. Knowing the batch weight and number of crystals the relative areas are easily estimated. Needless to say, these estimates are very crude. Comparisons of these numbers are made in Table III. For samples 4 and 5 the numbers of crystals were too large and the clumping too serious for significant estimates of total area to be made.

Another approach to the estimation of the effect of the actual surface layer of molecules lies in the use of macromolecular substrates or inhibitors which would be incapable of entering the crystal lattice. A synthetic block copolymer composed of glutamic acid and tyrosine was kindly supplied by Dr. G. Fasman. The particular preparation had a weight-average molecular weight of about 100,000 and a composition Glu:Tyr (95:5%). In spite of the COOH-terminal tyrosine residues this substance is not a substrate for carboxypeptidase. The strange behavior of this material and other related peptides has been described by Lehrer *et al.* (1965). It turns out that this particular block copolymer is a potent inhibitor of the enzyme in solution at low ionic strengths. Because of this it was tried as an indicator of surface activity for the solid forms of the enzyme. None of the columns of crystals referred to in the previous paragraphs showed any detectable change in activity when the copolymer was included in the assay solutions at a concentration giving 90% inhibition of the soluble enzyme. The lack of effect was not due to the chemical modification of the protein as columns of native crystals not treated with glutaraldehyde gave the same result. It was hoped that these observations indicated a very small contribution of the actual surface layer to the measured activity of the crystals. Unfortunately, the significance of the finding is uncertain since no inhibition was observed when the enzyme was in the form of an amorphous cross-linked mass. Here the surface:volume ratio was presumed to be huge and if the polymer were acting as an effective inhibitor, some decrease in activity of the preparation should have been observed. The results of these assays are shown in Table IV.

The substrate analog β-phenylpropionic acid is the most effective known inhibitor of carboxypeptidase in solution. The effect of this inhibitor on crystals

TABLE III: Effect of Crystal Size on the Apparent Activity of Carboxypeptidase-A.[b]

Sample	Dry Wt (mg)	No. of Crystals	Av Thickness[a] (mm)	Rel Area	Act. (ΔA_{570}/min)		Relative Activity per Unit Wt		Relative Activity per Unit Area	
					0.02 M	0.25 M	0.02 M	0.25 M	0.02 M	0.25 M
Large	1.05	225	0.058	1	0.056	0.142	1	1	1	1
Medium	0.25	540	0.028	0.51	0.032	0.053	2.4	1.6	1.1	0.7
Small	0.17	1550	0.012	0.56	0.053	0.101	5.8	4.4	1.7	1.2
4	0.065	—	0.0062	—	0.025	0.049	7.3	5.6	—	—
5	0.094	—	0.0027	—	0.038	0.053	7.6	4.2	—	—

[a] The values for average thickness are those directly estimated from the dry crystals. The changes in unit cell dimensions on drying have not been included. The actual average dimensions of the wet crystals used in the assays would be about 20% larger than those indicated. [b] Activities at two different substrate concentrations taken from Figure 3 or equivalent data for samples 4 and 5.

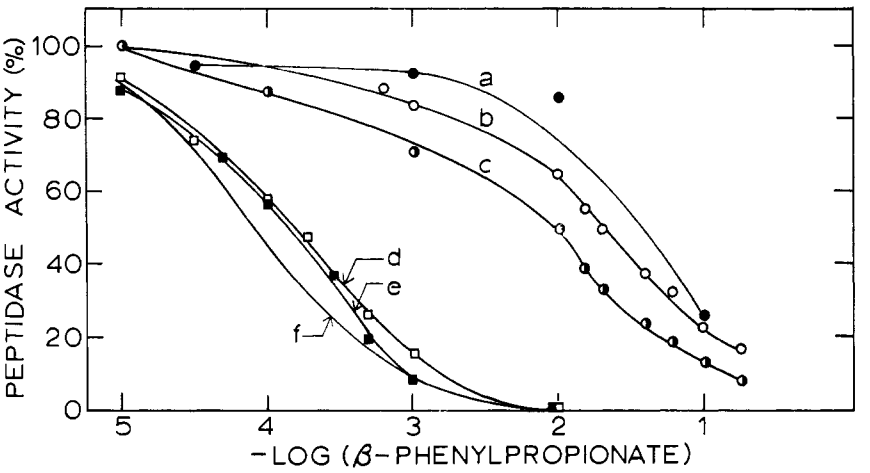

FIGURE 4: Inhibition of carboxypeptidase-A by β-phenylpropionate. The remaining peptidase activities, expressed as per cent of the uninhibited enzyme in the same form acting as a control, are plotted as a function of the negative logarithm of the inhibitor concentration. In addition to the inhibitor, the assay solution consisted of 0.02 M carbobenzoxyglycylphenylalanine, 0.02 M sodium veronal, pH 7.5. The temperature was 25°.

Curve	State of Enzyme	Ionic Strength	Enzyme (mg)
a	Large cross-linked crystals	1.5	1.05
b	Large cross-linked crystals	0.22	1.05
c	Small cross-linked crystals	0.22	0.169
d	Cross-linked amorphous	0.22	0.057
e	Native, solution	0.22	0.0013

The steady-state assay technique was used for assaying the crystalline and amorphous enzyme. The assay in solution, curve e, had a volume of 1.01 ml. Aliquots of 0.100 ml were taken at various time intervals. The activity was estimated as the change in absorbance at 570 mμ/min. The data for curve f was taken from Coleman and Vallee (1964). Their assay solution consisted of 0.01 M carbobenzoxyglycylphenylalanine, 0.1 M NaCl, 0.02 M sodium veronal, pH 7.5, and about 0.034 mg of enzyme, and the assay was performed at 0°.

TABLE IV: Effect of Block Copolymer Glu:Tyr (95:5) on the Activity of Carboxypeptidase-A in Various States.[e]

State of Enzyme	Act. (ΔA_{570}/min) Inhibitor (V_c)	Act. (ΔA_{570}/min) Inhibitor (V_i)	Residual Act. ($V_i/V_c \times 100$)	Inhibitor (mg/ml)
Large native crystals	0.076	0.078	102	0.072
Large cross-linked crystals[a]	0.063	0.061	97	0.072
Small cross-linked crystals[a]	0.053	0.057	108	0.072
Amorphous cross-linked	0.036	0.036	100	0.115
	—	0.035[b]	97	0.115
Native enzyme in solution	0.016	0.0032	20	0.072
Native enzyme in solution[c]	0.023	0.017	78	0.072
Native enzyme in solution[d]	0.011	0.007	6	0.072

[a] The columns of crystals were those used in assays shown in Figure 2. [b] Activity obtained after the same column of amorphous enzyme was treated with the inhibitor before assaying. [c] Assay solution contained 1.5 N NaCl. [d] Assay solution contained no added NaCl. [e] The steady-state assay was utilized in the activity measurements of crystalline and amorphous enzyme. The assay solution consisted of 0.02 M carbobenzoxyglycylphenylalanine, 0.2 N NaCl, 0.02 M sodium veronal, pH 7.5. The concentrations of the copolymer included in the assay solution are indicated in the last column.

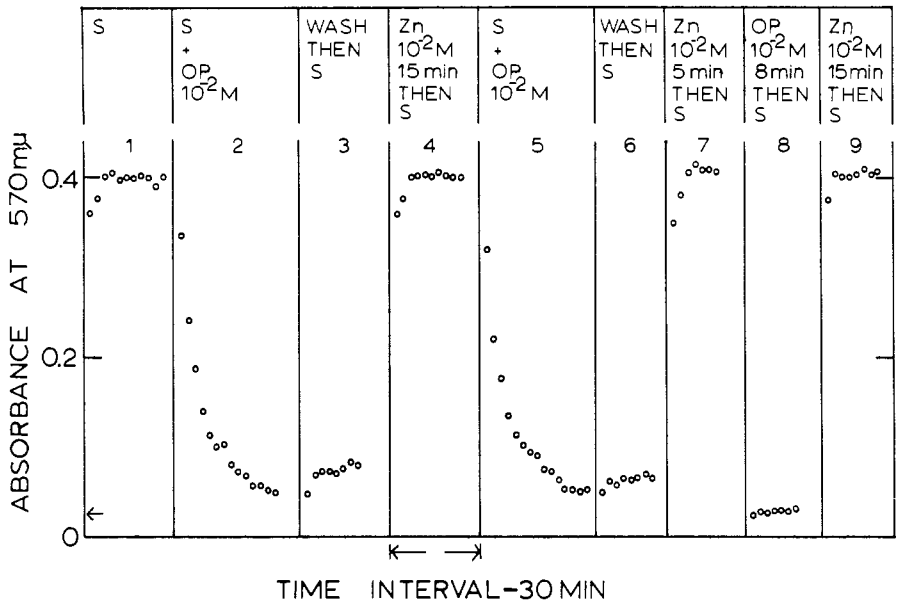

FIGURE 5: Inhibition of amorphous cross-linked carboxypeptidase-A by 1,10-phenanthroline. The column assay procedure was used. The actual ninhydrin color developed with successive drops from the column is shown. The standard assay solution contained 0.02 M carbobenzoxyglycylphenylalanine, 1.0 N NaCl, 0.02 M sodium veronal, pH 7.5. A stock solution of 1,10-phenanthroline was made in 1.0 N NaCl, 0.02 M sodium veronal, pH 7.5. The experiments were carried out consecutively from left to right during the course of one day. (The symbols S and OP stand for solutions of substrate and 1,10-phenanthroline, respectively.) (1) Standard assay; (2) assay as in 1 but in the presence of 0.01 M 1,10-phenanthroline; (3) the same assay as in 1 after washing for 30 min with deionized water; (4) the same assay as in 1 after the column was washed and treated with 0.01 M $ZnCl_2$, 1.0 N NaCl, 0.02 M sodium veronal, pH 7.5, for 15 min; (5–7) are experiments duplicating those shown in 2–4, respectively; (8) the same assay as in 1 after treatment with 0.01 M 1,10-phenanthroline for 8 min; (9) assay as in 1 after the column was washed and treated with zinc solution as in 4. The weight of the amorphous enzyme in the column was 0.11 mg. The arrow on the left ordinate shows the absorbance of the blank in the absence of any enzyme.

of the enzyme was studied. Varying concentrations of this substance were included in the assays. The data are shown in Figure 4. The inhibitor dissociation constants were taken as the concentration required to reduce the activity to one-half of the starting value. On this assumption the value obtained for the enzyme in solution, about 1×10^{-4} M, is comparable to those previously reported by Coleman and Vallee (1964), and by Elkins-Kaufman and Neurath (1949). The behavior of the cross-linked amorphous enzyme is indistinguishable from the soluble form. However, in the crystalline state the inhibition constant appears to be increased by about two orders of magnitude. This pronounced difference could not be attributed to the fact that the crystals were cross-linked. In a separate experiment, assay of a column of native crystals with a solution containing 0.02 M carbobenzoxyglycylphenylalanine, 0.01 M β-phenylpropionate, 0.02 M sodium veronal, pH 7.5, gave an activity of 51% that of the uninhibited control. This extent of inhibition was not affected by preincubation of the crystals with the inhibitor for 1 hr.

A variety of chelating agents (*e.g.*, 1,10-phenanthroline, α,α′-dipyridyl, and 8-hydroxyquinoline-5-sulfonic acid) inhibit native carboxypeptidase-A in solution by competing with the apoenzyme for its atom of zinc and removing it (Vallee, 1961). The zinc is removed without impairment of gross structure (Rupley and Neurath, 1960), and enzyme activity may be regained only by the addition of zinc or other transition metals (Coleman and Vallee, 1960). In view of this, the effect of 1,10-phenanthroline on carboxypeptidase-A in solid form was investigated.

The necessary precautions were carefully observed against contamination with adventitious metal ions contained in substrate and other solutions. All solutions and wash liquids were extracted at least three times with reagent grade CCl_4 containing 0.1% dithizone. Deionized water was obtained by passing glass-distilled water through a column of mixed IR-120 (H^+) and IRA-410 (OH^-) (Rohm and Haas). All reagents were stored in polyethylene bottles which were previously treated several times with a 1:1 mixture of HNO_3 and H_2SO_4.

The amorphous cross-linked enzyme was assayed using the column procedure. The data are shown in Figure 5 and the details of the experiment are given in the legend. The left box shows the initial activity of this particular column. Boxes 2 and 5 show the slow loss in activity during the assay that occurs

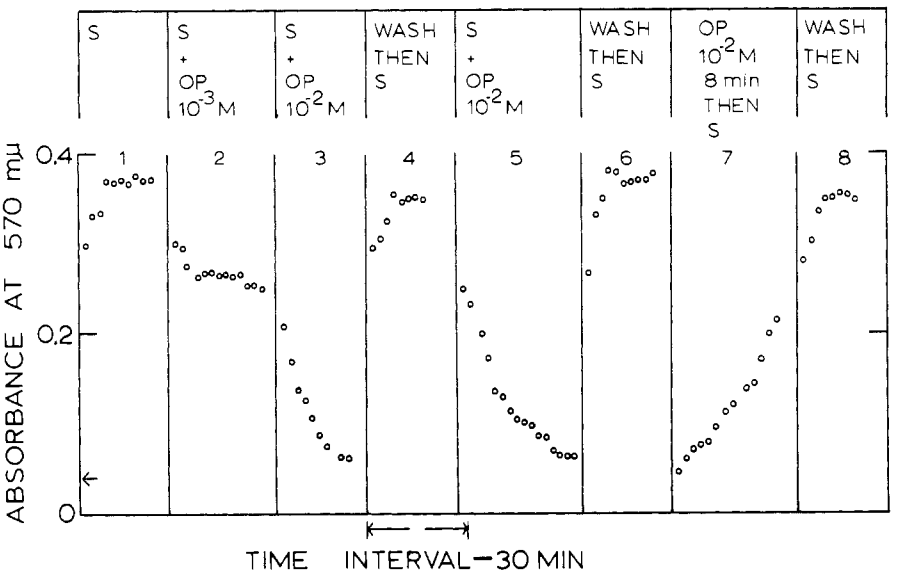

FIGURE 6: Inhibition of cross-linked carboxypeptidase-A crystals by 1,10-phenanthroline. The solutions and procedures were the same as those described in the legend to Figure 5. (1) Standard assay; (2) the same assay as in 1 but in the presence of 0.001 M 1,10-phenanthroline; (3) the same assay as in 1 but in the presence of 0.01 M 1,10-phenanthroline; (4) the same assay as in 1 after washing with water; (5) the same assay as in 3 after washing with water; (6) assay as in 1 after washing with water; (7) assay as in 1 after the column was washed with water and treated with 0.01 M 1,10-phenanthroline, 1.0 N NaCl, 0.02 M sodium veronal, pH 7.5, for 8 min; (8) assay as in 1 after washing with water for 8 min. The arrow indicates the substrate blank. The dry weight of the crystals in the column was 0.39 mg.

with the inclusion of 1,10-phenanthroline. After removal of the chelating agent, as in 3 and 6, a slight increase in activity is seen and the values are distinctly above the blank level. Addition of divalent zinc, as in 4, 7, and 9, results in complete recovery of the starting activity. When the 1,10-phenanthroline treatment is carried out in the absence of the substrate, the zinc is rapidly and effectively removed. Subsequent assay, 8, then shows no detectable activity. The 8-min treatment with the chelating agent in the presence of substrate would have reduced the activity only to 40–50% of the starting value. It is concluded that the zinc atom may be removed and reinserted in the amorphous enzyme very much as it is in solution with corresponding activity changes. The rate of the process is affected by substrate in both soluble (Felber *et al.*, 1962) and amorphous enzyme.

An equivalent experiment was carried out with a column of crystals previously cross-linked with 1% glutaraldehyde for 1 hr. The data are shown in Figure 6. Comparison of boxes 1 and 2 shows only a relatively small inhibition by 0.001 M 1,10-phenanthroline. With the chelating agent at 0.01 M, however, a slow loss of activity is observed very similar to that seen with the amorphous enzyme. The major difference occurs when the 1,10-phenanthroline is washed out. With no addition of zinc ions the activity returns to its initial value. This sequence is repeated in boxes 5 and 6. In 7 it is seen that, when the enzyme is inactivated by 1,10-phenanthroline and the chelating agent then washed out with the assay solution, the recovery of activity is slow, roughly a mirror image of the inactivation process shown in 3 and 5. After a final wash, 8, full activity is eventually regained. It is important to note that at no time during this entire sequence on the crystals has any zinc-containing solution been used. The presumption is then strong that the zinc atoms have not been removed from enzyme molecules in the crystal lattice.

Discussion

The intermolecular cross-linking produced by glutaraldehyde treatment has provided very useful material showing no solubility in any of the solvents of interest and having substantial residual enzymic activity. The chemical nature of the reaction is still obscure. Lysine in the only amino acid clearly implicated, but no derivative of lysine has been recognized in hydrolysates of the cross-linked material. The high reactivity of the alpha hydrogen atoms of aliphatic aldehydes, as well as the carbonyl function, make one suspect multiple side reactions and perhaps a variety of actual products. Fortunately, the useful properties of the material can be exploited without a detailed knowledge of the chemistry of the cross-linking reaction.

In the initial studies of the catalytic activity of crystals of ribonuclease-S, Doscher and Richards (1963) discussed the problems of diffusion limitation and of the estimation of the kinetic parameters for the enzyme in the crystal lattice. For the simple model of diffusion into a thick slab coupled with chemical

reaction, it was predicted that the rate, measured in the steady state as in the column assay, would be a linear function of the substrate concentration in the external liquid when this value was well below the true Michaelis constant for the crystalline enzyme. Conversely, at substrate concentrations well above the Michaelis constant, the rate would be expected to increase as the square root of the substrate concentration rather than be independent of it as in solution studies. The center parts of large crystals would never be exposed to the substrate and thus the estimated specific activity based on total weight of protein would depend on the size of the crystals. In this earlier work no investigation was made of the effect of crystal size.

The very smallest crystals of carboxypeptidase used in this study gave constant specific activities as noted in Table III. For these preparations diffusion limitation does not occur and the kinetic parameters may be derived as one would for solution studies. This constancy of specific activity is only obtained for crystals whose minimum dimension is of the order of 5 μ or less. The required dimension will, of course, depend on the rate of the reaction being studied. The same order of magnitude was predicted earlier for ribonuclease-S by the calculations of Doscher and Richards (1963). In their study of the reaction of azide with crystals of myoglobin, Chance *et al.* (1966) found no evidence of diffusion limitation with minimum dimensions in the range of 2–5 μ. The thickness of such crystals still represents many hundreds of protein molecules, and the catalytic or chemical behavior may properly be attributed to molecules in interior lattice sites.

TABLE V: Comparison of Specific Activities of Various Forms of Carboxypeptidase.[c]

	Sp Act. (ΔA_{570}/min mg of protein)		
	0.03 M[d]	1.5 M[d]	
Form of Enzyme	0.02 M[e]	0.02 M[e]	0.25 M[e]
Native			
Solution	140	300	210
Amorphous	2.2	X	X
Very small crystals	0.51	X	X
Cross-linked[a]			
Amorphous	(0.56)[b]	0.78	1.3
Very small crystals	0.25	0.41	0.56

[a] All materials referred to in this table were cross-linked in 1% glutaraldehyde for 1 hr at room temperature. [b] One column of amorphous enzyme was used to establish the specific activities at high ionic strength. A second column was used to establish the ratio of activities at low and high ionic strength. The weight of protein used in the second column was not measured and thus absolute specific activities could not be obtained from those data alone. [c] All assays carried out in 0.02 M sodium veronal buffer, pH 7.5, at the indicated substrate concentration. The high ionic strength solutions were made up with the required amount of NaCl. The symbol X appears where experiments are not possible. [d] Ionic strength. [e] Substrate concentration.

The specific activities under various conditions are summarized in Table V. There may be effects on activity caused by passing from solution to the solid states. There may be changes caused by the chemical modifications involved in the cross-linking process. Superimposed on these may be differences in response of the various forms to solvent composition (*i.e.*, ionic strength, pH, etc.). Some comparisons cannot be made. It is not possible to assay the solid forms of the native enzyme at high ionic strength, nor is it possible to assay the cross-linked material in solution. In spite of all these reservations, the answers to some questions can be seen in Table V.

Activity is reduced by a factor of about seventy in going from solution to amorphous enzyme and a factor of about 300 from solution to crystals. Diffusion limitation has been excluded. The changes are the result of some other effect. Limitations in structural flexibility come to mind, but there is no useful direct evidence for or against such an assumption. These changes in carboxypeptidase are much larger than those estimated by Doscher and Richards (1963) for crystalline ribonuclease-S acting on cytidine 2′,3′-phosphate. In this latter study even with crystals where diffusion limitation was clearly present the specific activity appeared to be only 10–40 times less than the solution value.

The cross-linking reaction *per se* causes a further reduction in activity by a factor of about 4 for the amorphous material and 2 for the crystals. This is presumably due to modification of specific functional groups. Restriction in pore size is also a possibility although this should only be seen in cases where there is some diffusion limitation, and should be absent for the smallest sizes. The epsilon amino groups of the lysine residues do not, however, appear to be of major importance for catalytic activity of the enzyme. Various chemical modifications of these groups have been reported by Riordan and Vallee (1963) and by Coombs *et al.* (1964). The relative effect of an increase in ionic strength is about the same for the soluble and crystalline forms of the enzyme. The change is considerably less for the amorphous material.

The substrate analog, β-phenylpropionic acid, is a potent inhibitor of the enzyme. In solution the inhibition data appear to fit the requirements for simple competition (Elkins-Kaufman and Neurath, 1948). Such behavior is often considered to have a direct geometrical interpretation with substrate and inhibitor being literally bound to the same part of the enzyme surface. In the present instance such an assumption is natural since a COOH-terminal phenylalanine residue on the peptide provides an excellent substrate, and the inhibitor differs from this amino acid only in the absence of the amino group. The first indication in

the present study that something curious was happening was the small effect that β-phenylpropionate had on the activity loss during the cross-linking reaction. The inhibitor was used at 0.01 M, a concentration 100 times the value of the inhibition constant as measured in solution. The enzyme should have been fully in the form of the complex. The protective effect of substrates or competitive inhibitors in the inactivation of enzymes by chemical modification are so common and so well known that it was surprising not to find such an effect in this case. Such protection has been found, in fact, with carboxypeptidase in solution (Walsh *et al.*, 1962; Riordan and Vallee, 1963). The answer seems to appear in the inhibition data in Figure 4. The apparent inhibition constant for the crystals is about 0.01 M. Thus only one-half the enzyme in the crystals is in the form of a complex at an inhibitor concentration of 0.01 M, and the inhibitor could not effect the inactivation rate by more than a factor of roughly two. This is about the magnitude required to explain the data in Figure 2.

The existence of substrate inhibition for peptidase, and especially esterase, activity in the kinetics of carboxypeptidase action has been known for a long time (Snoke and Neurath, 1949; Lumry *et al.*, 1951). There appears to be no mechanism for this phenomenon which requires the assumption of less than two binding sites for the substrate. This fact in itself presaged the complicated kinetic picture for this enzyme that continues to unfold (Lumry *et al.*, 1951; McClure *et al.*, 1964; Carson and Kaiser, 1966; Whitaker *et al.*, 1965). The solution curve in Figure 3 shows typical substrate inhibition behavior. The fully inhibited enzyme must still have considerable activity or the curve would approach zero activity at high substrate concentrations. The data for the solid enzymes are replotted in double-reciprocal form in Figure 7. Marked curvature is observed for all the samples except the "large" crystal set. Thus extra substrate interaction sites are still present even though the curves in Figure 3 for the solids are qualitatively different than the one for the soluble form. The curved double-reciprocal plots indicate that the rate equation for this system must have terms in higher powers of the substrate concentration. A very general mechanism involving only a monomeric form of the enzyme and various substrate binding sites would be the following.

$$C_0 \equiv \text{free enzyme}$$
$$C_1 \rightleftharpoons C_0 + S$$
$$C_2 \rightleftharpoons C_1 + S$$
$$\vdots$$
$$C_{n-1} \rightleftharpoons C_{n-2} + S$$

$$C_0 + S \rightleftharpoons C_0S \longrightarrow C_0 + P$$
$$C_1 + S \rightleftharpoons C_1S \longrightarrow C_1 + P$$
$$C_2 + S \rightleftharpoons C_2S \longrightarrow C_2 + P$$
$$\vdots$$
$$C_{n-1} + S \rightleftharpoons C_{n-1}S \longrightarrow C_{n-1} + P$$

The rate equation for this mechanism will have the form of the general polynomial

$$-\frac{d(S)}{dt} = V = \frac{A_1(S) + A_2(S)^2 + A_3(S)^3 + \ldots + A_n(S)^n}{1 + B_1(S) + B_2(S)^2 + B_3(S)^3 + \ldots + B_n(S)^n} \quad (1)$$

where $A_1 \ldots A_n$, $B_1 \ldots B_n$ are appropriate combinations of rate constants for the individual steps. This scheme is similar to the somewhat more restrictive mechanism proposed by McClure *et al.* (1964) to account for the substrate inhibition of carboxypeptidase by hippuryl-DL-β-phenyllactate. Attempts at curve fitting for the data presented in this paper indicate that n cannot be less than 3 in most cases. Sets of constants which give reasonable fits were used to calculate the curves drawn through the points in Figure 7. No claim for uniqueness can be made since with so many parameters a large number of sets could have been chosen. For simplicity the turnover of the complex C_1S was assumed to be zero in order to maximize the curvature introduced by the cubic term. It is clear that there are sites other than the catalytic site which interact with the substrate and which may modify the behavior of the catalytic site. The number of these modifier sites is not less than two.

Whether the modifier sites in the crystals of the enzyme should be considered inhibitory is unclear at this time. If all the complexes, C_iS, turn over at the same rate, then one would not be inclined to call it substrate inhibition. The V–(S) curves would be sharpened and the double-reciprocal plot curved to an extent dependent on the values of n and the various constants. On the other hand, the turnover of the higher complexes may be much less than that of C_1, implying marked inhibition, but by proper choice of constants the curves can be made indistinguishable in shape from those of the first case. A very marked increase in the substrate inhibition effect could, in principle, explain the large drop in observed turnover noted on going from solution to the solid forms. The present experiments cannot resolve the dilemma.

The straight line obtained for the large crystals is assumed to be fortuitous and not the result of simple Michaelis–Menten kinetics. These crystals presumably retain the substrate binding complications considered above and in addition have superimposed the problems of severe diffusion limitation.

If β-phenylpropionate is in fact bound to the same part of the catalytic site as the phenylalanine portion of the substrate, then some very complicated changes in rate constants must occur between the various forms. The amorphous and crystalline states are very similar with regard to increasing substrate concentration, the maximum specific activities differ by a factor of 4 or less, and both are different from the soluble form. However, in the inhibition by β-phenylpropionate the amorphous and soluble forms are indistinguishable

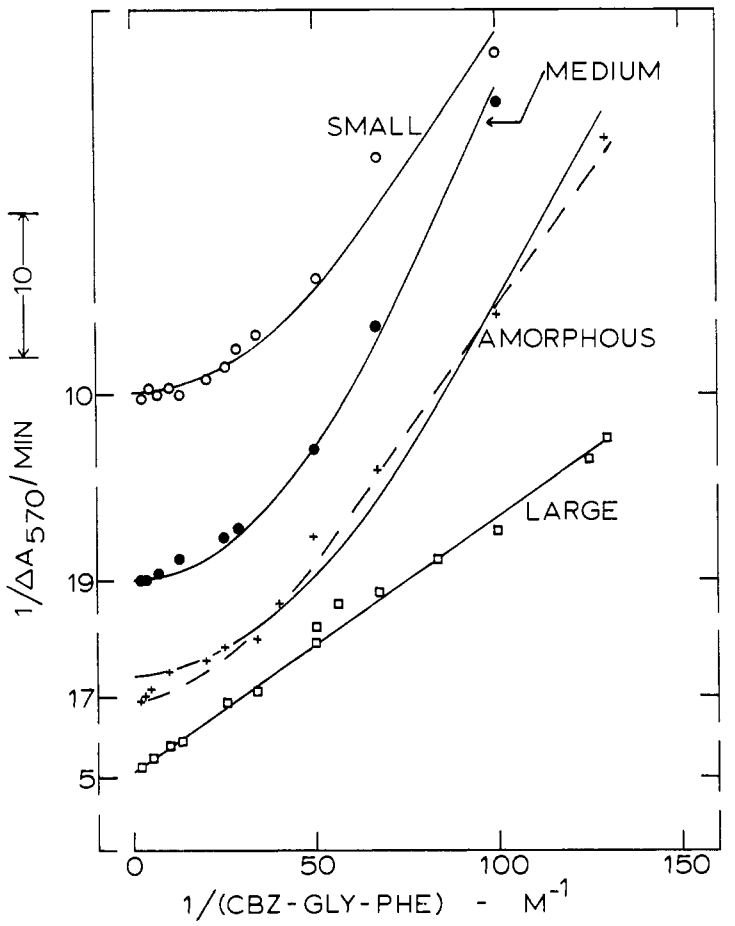

FIGURE 7: Double-reciprocal plots of the data in Figure 3. The curves have been computed from eq 1 with the following coefficients.

	A_1	A_2	$A_3 \times 10^{-3}$	B_1	B_2	$B_3 \times 10^{-4}$
Small	2.53	0	16.2	25.3	639	16.2
Medium	1.65	0	16.4	31.5	990	31.2
Amorphous						
Solid line	1.95	0	25.2	36	1300	46.6
Dashed line	2.64	115	0	43.5	1890	0
Large						
Either	5.78	0	0	31.8	0	0
Or	5.76	0	5.76	31.7	1000	3.17

The straight line for the large crystals is fitted equally well by either set of coefficients, the first simple Michaelis–Menten kinetics, the second implying multiple substrate binding sites. The value of the ordinate near the intersection for each curve is indicated. The scale of the ordinate for each curve is the same but the origins have been adjusted to avoid overlap.

while the crystals differ by a factor of 100 from the other two.

It should be noted that the same general equation and mechanism given above can also provide V–(S) curves with a sigmoid shape. This effect has been noted recently by Kaiser *et al.* (1965) in a study of the hydrolysis of *O*-hippurylglycollate. Addition of *N*-carbobenzoxyglycine abolished the sigmoid shape. It would appear that these phenomena as well as the ones reported in this paper can be explained by multiple binding sites for substrates and similar modifiers on a single macromolecule with only one catalytic site.

The intimate correlation between the zinc atom and the enzymic activity of carboxypeptidase has been carefully documented by Vallee and his colleagues (Vallee *et al.*, 1960; Vallee, 1961). A number of chelat-

ing agents remove the zinc with a corresponding loss in activity. The data on the treatment of the cross-linked amorphous enzyme (Figure 5) are remarkably similar to the solution experiments. The chelating agent, 1,10-phenanthroline, causes a loss of activity which is completely restored by the addition of zinc salts. The slow activity loss when chelation is carried out in the presence of substrate implies substrate protection, again in agreement with the solution data (Felber *et al.*, 1962).

The treatment of crystals of carboxypeptidase with 1,10-phenanthroline gave very different results. Loss of activity was observed when the assays were carried out in the presence of the chelating agent. However, full activity was recovered merely by washing the crystals with deionized water. No addition of zinc was required to effect the recovery. The implication was very strong that the zinc atom had not been removed. The metal analyses reported in the accompanying paper confirm that in the particular solvent used in the experiments shown in Figure 6, the zinc was not removed (Bishop *et al.*, 1966).

Inhibition by chelating agents without removal of the metal has been shown for other zinc metalloenzymes such as liver alcohol dehydrogenase (Hoch *et al.*, 1958). In such cases there is a ternary complex between the apoenzyme, the metal ion, and the chelating agent. In the work with carboxypeptidase in solution such a complex was carefully sought after but no evidence for it was found (Felber *et al.*, 1962). Such a complex presumably does occur in the case of the crystals. Information on the stoichiometry of the complex from direct binding data or spectrophotometric studies on crystal suspensions is not yet available.

The same effect of substrate in slowing the rate of the 1,10-phenanthroline inhibition, already seen with the soluble and amorphous enzyme, is also clear in Figure 6 for the crystals. In the reverse procedure, when assays are started with solutions not containing 1,10-phenanthroline on crystals initially fully inhibited, activity is recovered but quite slowly. It appears that the removal of complexing agent from the metal atom is much slower in the presence of substrate than in its absence. (For carboxypeptidase this behavior can only be observed in the crystals where the ternary complex appears to exist.) It seems likely that the substrate interacts with some part of the protein not involving the zinc directly but influencing its interaction with the chelating ligand. In the absence of such an interaction it is hard to imagine why the presence of substrate molecules in solution should affect the rate of dissociation of the 1,10-phenanthroline. Thus the behavior of this inhibitor provides a further indication of multiple substrate binding sites.

It is clear that the extension of studies such as those reported here can define the properties of enzymes in lattice sites in crystals in the same manner as is commonly done in solution. Detailed structures and structural changes will frequently be available from diffraction data for correlations. What is still unclear in detail is the relation of the crystal and solution structures. For carboxypeptidase it is interesting that all of the phenomena observed in solution are also seen in the solid forms. However, it is also true that in all cases the behavior is modified at least to some extent. There are very few guidelines by which to assess the structural significance of these differences. In organic chemical studies of catalysis in model systems very large rate changes can frequently be produced by what might be considered small changes in structure. Such changes, in the fraction of an Angstrom unit range, might easily not be detectable at the resolution projected for most current protein crystallographic investigations. In this sense the solution and crystal structures would be the "same" while their chemical behavior differed markedly. From this point of view the very low turnover number observed for crystals of carboxypeptidase does not necessarily imply a large conformational change.

Some of the differences between solution and crystal behavior may be the result of local steric problems produced by the mode of packing in the lattice. Most protein crystals are polymorphic. Comparative studies of the properties of a single protein in crystals with different space groups should help to eliminate this particular difficulty.

Acknowledgments

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