The Removal and Exchange of Metal Ions in Cross-Linked Crystals of Carboxypeptidase-A*

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ABSTRACT: The metal content of small batches of cross-linked carboxypeptidase crystals was measured by X-ray fluorescence spectroscopy. The crystals were mounted in a flow cell which permitted simultaneous enzymic activity assays. Diffusion of both the free zinc cation and its 1,10-phenanthroline complex into and out of the crystals was demonstrated. At low ionic strength or in 1.0 M NaCl, 1,10-phenanthroline caused a decrease in zinc content of less than 10% while under comparable conditions with 0.5 M $NaNO_3$ as the electrolyte zinc removal by the chelating agent was essentially complete. Activation of the crystals was noted when cobalt replaced zinc and also when cobalt was bound to the protein in addition to the atom of zinc. A decrease in activity was noted in the presence of mercuric ion. A crystal batch with less than 0.1 g-atom of zinc and more than 4 g-atoms of mercury/mole of protein had more than 30% of the peptidase activity shown initially by the same batch before removal of the zinc. Even after the zinc was put back 1–2 moles of mercury could not be removed by washing. This final preparation, still containing the extra mercury, was somewhat more active than the starting zinc enzyme.

The importance of the zinc atom and the effect of substituting other divalent cations on the enzymic activity of carboxypeptidase-A have been studied extensively by Coleman and Vallee (1960, 1961). The use of this binding site has been important in preparing isomorphous derivatives for the X-ray diffraction program being carried out by Lipscomb and his associates (Hartsuck *et al.*, 1965). As part of a study of the catalytic activity of this material in the crystalline state, some preliminary observations on the interaction of cations with the protein have been made and are reported in this paper. This work has a direct bearing on the diffraction study since many samples for the latter are prepared by "soaking" crystals in various heavy metal salt solutions. It is also of interest to correlate the enzymic effects of metal exchange in solution as compared to the crystalline state.

Carboxypeptidase has been shown to be catalytically active in the crystalline state (Quiocho and Richards, 1964). A more detailed investigation of the kinetic behavior of the crystals has been reported in the previous paper (Quiocho and Richards, 1966). It was noted there that treatment of the crystals with 1,10-phenanthroline did not cause irreversible loss of activity. Presumably the zinc atom was not removed when the protein was in the crystal lattice. It was necessary to establish this point by direct analysis, and to determine whether the zinc–1,10-phenanthroline complex could enter or leave the crystal lattice. A convenient analytical technique for this purpose was X-ray fluorescence. Since the method is largely nondestructive, a series of metal-exchange experiments could be conveniently studied in a single batch of crystals.

Materials and Methods

The preparation of cross-linked carboxypeptidase crystals, the substrate solutions, and the column assay procedure have been described in the preceeding paper (Quiocho and Richards, 1966). Crystals were cross-linked at room temperature in 1% glutaraldehyde for 1 hr unless otherwise indicated. All metal salts used were analytical reagent grade. Solutions intended to be metal free were extracted with 0.1% dithizone in carbon tetrachloride several times.

X-Ray Fluorescence Measurements. The general principles and a number of applications of this technique for elementary analysis are given in texts such as those by Birks (1959) and Liebhafsky *et al.* (1960). Specific applications to biochemical problems are covered in the review by Natelson and Whitford (1964). The preliminary studies reported in this paper were done on standard commercial equipment with only minor modifications. The equipment design was far from optimal, but extensive development did not appear warranted during this initial survey.

The exciting X-ray beam was supplied by a tungsten target tube operated at 50 kv, the maximum voltage

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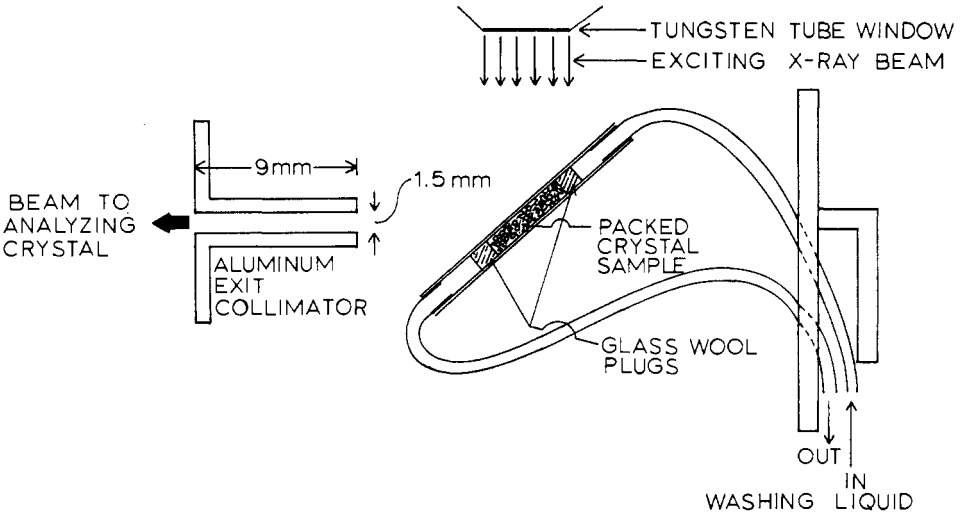

FIGURE 1: Schematic diagram of sample chamber for X-ray fluorescence measurements. The drawing is not to scale and the supporting members of the sample housing are not shown. The X-ray tube is above the sample and the spectrometer to the left of the figure.

available from the General Electric XRD-5 power supply. Since the power supply was serving a second tube used for diffraction cameras, the maximum current available for the tungsten tube was 16 ma when both systems were operating simultaneously. The tube was mounted on a General Electric SPG-4 spectrogoniometer. A lithium fluoride crystal was used as analyzer for the fluorescent beam. The beam diffracted from the lithium fluoride crystal passed to a scintillation detector. The detector output was fed to a Picker Nuclear Corp. Spectroscaler III, equipped for pulse height analysis, and also to a ratemeter and recorder.

A schematic diagram of the sample chamber is shown in Figure 1 without the supporting parts of he holder. The cross-linked crystals were packed as tightly as possible between two glass wool plugs in a polyethylene tube (PE 160, i.d. 1.1 mm). The sample tube was fitted with smaller inlet and outlet tubes and all were completely liquid filled at the time of loading. The composition of the liquid could be changed at will during measurement.

The sample tube was mounted and held so that the crystals were all within the angular limits of the exit collimator and Soller slit in front of the detector. All radiation reaching the analyzing system had to pass through the cylindrical exit collimator. The diameter of the sample tube and collimator were chosen so as to get all of the sample within the collimator field and as little of anything else as possible. The noncrystal material consisted of the appropriate length of polyethylene tubing and small portions of the glass wool plugs, and the liquid filling the lumen of the tube and surrounding the crystals. All of this material, including the crystals themselves, contributed to the scattering and produced a background at all wavelengths much higher than one would have liked for accurate estimation of trace components. All of the material also gave off fluorescent X-rays of wavelengths characteristic of the elements represented. With the exception of the metals of interest in this study, all the elements in the crystals, liquid, and mount were of such low atomic number, and the fluorescent X-rays thus of such long wavelength, that absorption was extreme and such fluorescence never reached the detector.

Measurements were made at Bragg angles for the analyzing crystal corresponding to the weighted $K\alpha$ doublets for zinc (1.436A) and cobalt (1.790A) and to the $L\beta_1$ line for mercury (1.049A). Measurement in the sample chamber of solutions of known concentration, 0.01 M and below (but without crystals), gave a linear relation between concentration and an observed count rate above background. Absorption varied markedly with wavelength and was thus different for each of these metals. However, the geometry of the system was fixed and the absorption a constant effect which was removed by calibration with the solutions of known concentration. The absorption coefficient for protein crystals is not very different from that of pure water and thus the calibrations made with only solutions in the tube have been assumed to apply in the presence of the protein crystals. (The correction for volume actually occupied by the protein is discussed below.) The only exception to this statement is for measurements made in the presence of high concentrations of sodium chloride. The change in absorption coefficient between water and 1.0 M NaCl is sufficient to cause a substantial change in the count rate for standard zinc and cobalt samples. Only a few measurements were made in high salt and special calibration curves were not run.

Changes in absorption owing to changes in metal content of the samples were also considered. From the known mass absorption coefficients, the most

serious combination encountered was the measurement of zinc radiation in the presence of mercury. Even in this case for the maximum amount of mercury present in any measurement reported, the error in zinc content owing to absorption was less than 10% of the observed value. In view of the accuracy required for the purposes of this survey no absorption corrections have been applied.

The volume of the lumen of the polyethylene tube "seen" by the exit collimator was measured in the complete set up. With the tube initially clean and dry the background count rate at the zinc Kα position was established. An "Agla" micrometer syringe filled with 0.01 M $Zn(NO_3)_2$ was connected to the "out" liquid delivery tube. The solution was run backwards through the system. The syringe reading was noted when the background rate just started to increase. The flow was continued until no further change in count rate occurred and the syringe reading was again noted. In repeated tries, the volume estimate was 10 $\pm$ 1 μl. This figure was used to convert the liquid calibration data to counts per unit weight of metal, a number directly applicable to the crystal measurements.

The metal ion of interest can be (1) in the flowing liquid in the interstices between the packed crystals, (2) in the solvent-containing portion of the crystal lattice (42% of the total crystal volume), or (3) bound to the protein in the crystal lattice. Any measured value will represent the sum of all of these contributions. Salt ions free in the crystal lattice will be in equilibrium with those in the external liquid. General experience indicates that the concentrations will be comparable, with that in the crystal liquid tending to be somewhat lower owing to excluded volume effects. Thus, if the flowing liquid contains a very small or zero concentration of metal ion, all the metal found by analysis must be bound to the protein. In measurements removed from the isoelectric point counter ions of the appropriate sign will be required. If only electrostatic effects are involved the efficiency of the column percolation procedure should ensure that such ions come from the salt solution most recently in contact with the crystals. The isoelectric point of the protein in the crystal lattice is unknown. In solution pI for the native enzyme is 6.2. Modification of the amino groups would move this further into the acid region. Since most experiments were performed at pH 7.5, a number of cationic counter ions must always have been present.

At the termination of a series of experiments, the crystal sample was exhaustively washed with water, removed from the plastic capillary, and dried to constant weight on a tared coverslip over anhydrous $CaCl_2$ *in vacuo* at room temperature. The weight of salt-free protein used in the various experiments was in the range 1.5–3.0 mg. Several estimates were made of the zinc content of native crystals using the zinc calibration data from the standard solution and assuming a molecular weight of 34,000 for the protein. For five determinations the average value

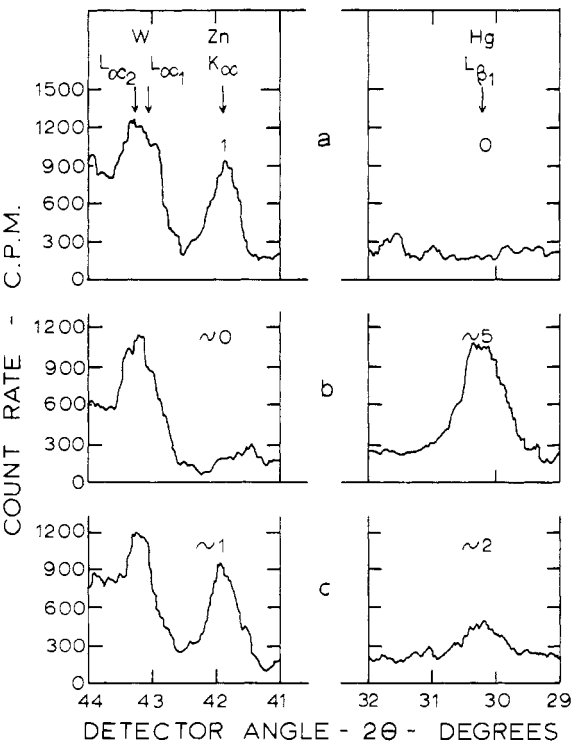

FIGURE 2: Limited 2-θ scans showing the peak positions for zinc and mercury. The detector angle was changed at a rate 2°/min. The ratemeter was set at 3000-cpm full scale with a time constant of 0.3 sec. The actual mercury $L\beta_1$ line is at 30.19°, the Zn Kα doublet at 41.80°, and the background tungsten $L\alpha_1$ and $L\alpha_2$ lines at 43.02 and 43.36°. The estimated stoichiometry of metal to protein from static counts at the peaks in these examples is listed over the peak position. The details of the treatment of this particular crystal batch is given in Table III.

for gram-atoms of zinc per mole of enzyme was 1.0 with a standard deviation of $\pm$0.06 and a maximum deviation of 0.2. The value giving this maximum error was based on measurements of a protein sample weighing only 0.2 mg.

Where it was necessary to distinguish between protein-bound metal ion and ions free in solution, the total sample volume, 10 μl, was reduced by the volume of protein calculated subsequently from the measured weight and a partial specific volume of 0.75 ml/g. This estimated liquid volume was assumed to have a uniform concentration equal to that of the flowing liquid supply. The counts to be expected from this free metal were taken from the solution calibration data corrected for the volume ratio. These calculated "liquid" counts were subtracted from the measured counts to get the "bound" counts. This latter figure could be converted to gram-atoms of metal per mole of protein by again using the measured protein weight.

Some typical traces of count rate as a function of Bragg angle are shown in Figure 2. The first scan, on freshly prepared cross-linked crystals, shows no counts

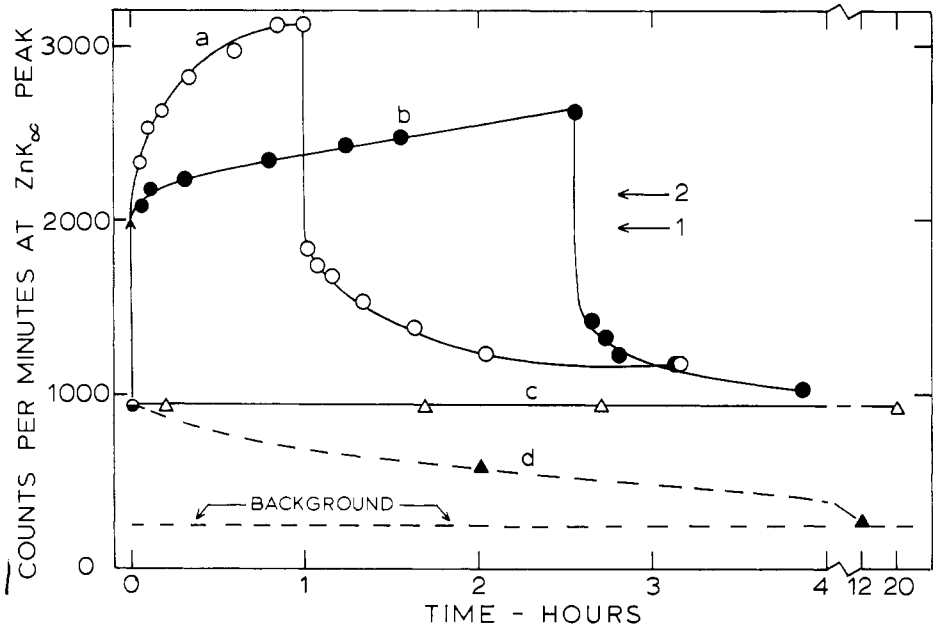

FIGURE 3. Diffusion of Zn^{2+} and $Zn(OP)_3^{2+}$ ions into and out of crosslinked crystals of carboxypeptidase-A. The effective volume of the sample tube was 7 μl. When filled with a 0.01 M $Zn(NO_3)_2$ solution, the measured fluorescence at the zinc peak was 1400 cpm above the background level of 250 cpm obtained with pure water. The packed crystal sample gave 700 counts above background as seen in the figure. The volume occupied by the protein (1.2 mg) was about 1 μl. The crystal solvent volume was about 1 μl and the liquid volume external to the crystals was, therefore, about 5 μl. In curve a, open circles, 0.01 M $Zn(NO_3)_2$ was flowed over the crystals. The rapid rise of about 1000 cpm expected for filling the 5-μl liquid space external to the crystals is shown by the arrowhead on the curve of observed count rate. At 1 hr the liquid was replaced with pure water. The rapid drop of about 1000 cpm reflects the flushing of the external liquid followed by the slower outward diffusion of the zinc salt from the crystal. In curve b, filled circles, the same crystal sample was exposed to a 0.01 M $Zn(OP)_3(NO_3)_2$ solution. At 2.6 hr, the liquid was changed to water. The count rate returned to the starting level after a number of hours of washing. In curve c, open triangles, the crystals were washed for 20 hr with 0.01 M 1,10-phenanthroline. In curve d, closed triangles, addition of 0.5 M $NaNO_3$, 0.02 M veronal buffer, pH 7.5, to the 1,10-phenanthroline solution resulted in removal of the zinc. The actual experiment in this case was done on a separate batch of crystals with different background and initial count rates. The numbers have been scaled to fit this graph for easy comparison. The horizontal arrow labeled 1 represents the expected count rate if no zinc entered the crystal lattice. The arrow labeled 2 is at the level expected if the concentration of zinc in the crystal liquid was equal to that in the external liquid, but with no "extra" zinc bound to the protein.

above background in the region of the mercury line and a prominent zinc peak just before the background peak owing to a characteristic tungsten line in the radiation from the exciting X-ray beam. After removal of the zinc atom by exposure to a solution containing mercuric ion, the second scan was obtained. The small peak at about 41.5° is part of the background fluctuation and is not actually at the Zn Kα position of 41.80°. Attempts to flush out the mercury and replace the original zinc resulted in scan 3. It can be seen that the zinc peak is almost the starting size but a residual mercury peak is still clearly evident. At the time the scans were made all detectable free metal ions had been flushed out of the system. The details of the crystal treatment prior to these scans is shown in Table III. With the exception of occasional traces as a check, the tabulated data were collected as consecutive fixed time counts at the appropriate 2-θ position. All values are reported as counts per minute. Monitor counts were frequently taken for shorter periods but all entries containing estimates of stoichiometry are counts actually accumulated over a 1-min period unless otherwise indicated. An estimate of the standard deviation of any individual measurement is the square root of actual counts taken, as usual. Such reliability estimates are not listed individually in the tables.

The sensitivity of cross-linked crystals of carboxypeptidase to radiation damage in the fluorescence instrument has not yet been examined in detail. During these initial experiments the radiation exposure was kept as low as possible. The short count times frequently used severely limited the potential analytical accuracy of the method and the limits of error suggested for the reported analyses are correspondingly large. It is probable that no severe radiation effects were incurred since activity values at the end of a cyclic series of metal exchanges and measurements were generally close to the original values. In retro-

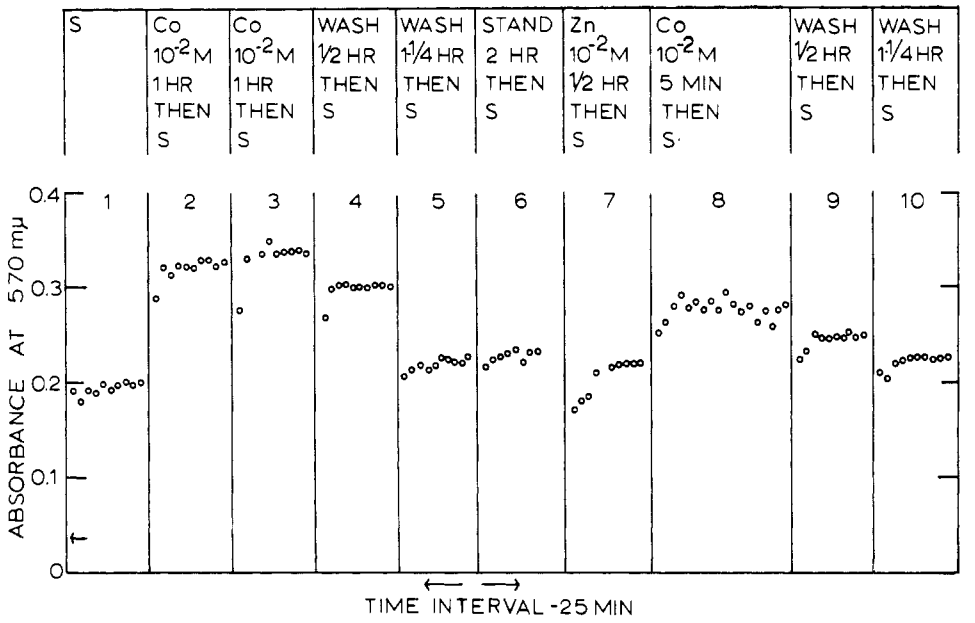

FIGURE 4: Activation of cross-linked carboxypeptidase crystals by cobalt. The column assay procedure was used. The actual ninhydrin color developed with successive drops from the column is shown. The assay solution is given in the heading to Table I. The arrow at the left ordinate shows the assay blank value. The cobalt solution contained $CoCl_2$ 0.01 M, NaCl 1.0 M, Tris 0.01 M, pH 7.5. After each assay the column was washed with deionized water for 10 min before the next solution was applied. The indicated wash periods also used distilled water but for longer periods. The headings in the boxes give the treatment of the column just prior to the assay shown directly below. The symbol S stands for the substrate solution used in the assay. The experiments were carried out consecutively as listed. At the end of this series the dry weight of the protein was found to be 0.18 mg.

spect, longer count times could probably have been employed without danger.

Results

Preliminary analyses indicated that crystals treated with 0.01 M 1,10-phenanthroline showed no loss of zinc. A possible trivial reason for this would have been the inability of the Zn–(1,10-phenanthroline)$_3^{2+}$ complex to leave or enter the crystal lattice for purely steric reasons. The data in Figure 3 show that this is not the case. This particular batch of crystals gave a total count rate at the zinc position of about 950 cpm. On correction for background, the residual counts corresponded very closely to 1 g-atom of zinc/mole of protein. After the rise in count rate owing to Zn^{2+} in the liquid external to the crystals, the filling by simple diffusion of the internal solvent could only have contributed an additional 200 cpm (arrow 2). The much larger rise observed can only be explained by binding. At the highest value shown in the figure this "extra" zinc corresponds to 1.6 g-atoms of Zn^{2+} bound/mole of protein. This extra zinc was rapidly washed out when the zinc salt was removed from the flowing liquid. The experiment was repeated with a solution containing the same concentration of zinc but in the form of the 1,10-phenanthroline complex. The very rapid rise in count rate is again seen, followed this time by a much slower diffusion process as would be expected for the large complex ion. The free diffusion coefficients for this complex would be considerably less than that for the zinc cation. The complex is probably also large enough to be affected by restriction due to the small pores in the crystals. This latter effect would serve to enhance the difference in the apparent diffusion coefficients of the two forms of the metal ion in the solid. When this same crystal batch was treated with a 0.01 M solution of 1,10-phenanthroline, there was a barely detectable change in the count rate at the zinc peak over a period of 20 hr. It is very clear that, under these conditions, zinc is not removed from the protein crystals, thus confirming the conclusions already drawn from activity measurements.

The experiments reported in Figure 3a–c were all carried out at low ionic strength. Experiments in the presence of 1.0 N NaCl gave similar results. Again long periods of exposure to the chelating agent failed to lower the zinc content of the crystals. This behavior is changed in the presence of sodium nitrate. When a solvent consisting of 0.01 M 1,10-phenanthroline, 0.5 M sodium nitrate, 0.02 M sodium veronal, pH 7.5, was used, the zinc content dropped to 56% of the starting value in 2 hr (Figure 3, curve d). Further extraction for twelve hours reduced the zinc to a barely detectable level. The same nitrate-containing salt solution, but with the chelating agent omitted,

TABLE I: Attempts at Direct Exchange of Cobalt for Zinc.

Carboxypeptidase crystals, cross-linked for 1 hr in 1% glutaraldehyde, mounted in a flow tube in the X-ray fluorescence spectrometer. The series of operations outlined below were carried out sequentially on the single column of crystals. The total cumulative time of exposure of the crystals to the exciting X-ray beam in the fluorescence measurements was 40 min. Estimated errors for columns 5 and 6, indicating stoichiometry, are ±0.1 g-atom/mole for zinc and ±0.2 g-atom/mole for cobalt.

Assay in chloride: substrate solution contained carbobenzoxyglycylphenylalanine 0.02 M, NaCl 1.0 M, sodium veronal 0.02 M, pH 7.5. The initial activity = 100% = 0.200 ΔA_{570}/min.
Chloride–cobalt: NaCl 1.0 M, $CoCl_2$ 0.01 M, sodium veronal 0.02 M, pH 7.5
Nitrate–cobalt: $NaNO_3$ 0.5 M, $CoCl_2$ 0.01 M, sodium veronal 0.02 M, pH 7.5
Dilute buffer: sodium veronal 0.001 M, pH 7.5.

Flowing Soln or Operation on Column of Crystals	Liquid Flow Time (per expt), min	Measured Counts at Position of Kα Lines Including Background (cpm) Zn	Co	Estd Stoichiometry (g-atoms of metal bound/mole of protein) Zn	Co	Peptidase Act. (% initial)
Flow tube						
H_2O	—	145	40			
0.01 M $Zn(NO_3)_2$	—	1345	—			
0.01 M $CoCl_2$	—	—	327			
Dilute buffer	0	1075	40	1.2	0	100
Chloride–cobalt solution	5		557[b]			
	32	735[b]	810[b]			
	56	667[b]	880[b]			
Assay in chloride	18					120
Wash, dilute buffer	60	760	270	0.8	1.3	
Assay in chloride	14	782[b]	120[b]			100
Nitrate–cobalt solution	10	731				
	16	703	1200			
	30	685				
	61	619				
	90	539	1380			
Wash, dilute buffer	10	557	1040	0.6	5.5	
Assay in chloride	15					110
Wash, dilute buffer	60	592	363	0.6	1.8	
Nitrate–cobalt solution	960	377	1576	0.3	7.2[a]	
Assay in chloride	15					105
Wash, distilled H_2O	15	384	680	0.3	3.6	
$ZnCl_2$, 0.01 M	60	>3000	187	>3	0.8	
Wash, distilled H_2O						
Assay in chloride	15					85
Wash, distilled H_2O	900	2600	225	>3	1.0	
Wash, 1.0 N NaCl	120	1250				
Wash, dilute HCl pH 3.5	30	975		1.1		
Assay in chloride	14					115
Wash, distilled H_2O	20	891	98	1.0	0.4	

[a] Count data corrected for "free" cobalt in liquid spaces to get estimate of "bound" cobalt. [b] These values have not been corrected for the absorption due to the 1.0 M NaCl in the sample liquid.

did not cause any significant removal of zinc from batches of fresh crystals.

The ability of 1,10-phenanthroline to remove zinc in the presence of nitrate ion is a clear indication that the chelating agent is capable of entering the crystal lattice. The diffusion experiment shown in Figure 3,

TABLE II: Preparation of "Cobalt" Enzyme.

A new column of cross-linked crystals was used. The procedure is described in the heading to Table I with additional solutions. The total cumulative time of exposure of the crystals to the exciting X-ray beam in the fluorescence measurements was 11 min. Error estimates the same as in Table I.

Nitrate-1,10-phenanthroline: 1,10-phenanthroline 0.01 M, $NaNO_3$, 0.50 M, sodium veronal 0.02 M, pH 7.5.
Dilute cobalt: $CoCl_2$ 10^{-4} M, sodium veronal 10^{-4} M, pH 7.5
Assay in chloride dilute cobalt: standard peptidase assay solution plus $CoCl_2$ 10^{-4} M.
Dilute zinc–cobalt: dilute cobalt solution plus $ZnCl_2$ 10^{-4} M, all adjusted to pH 6.8.

Flowing Soln or Operation of Column of Crystals	Liquid Flow Time (per expt), min	Measured Counts at Positions of Kα Lines Including Background (cpm) Zn	Co	Estd Stoichiometry (g-atoms of metal bound/mole of protein) Zn	Co	Peptidase Act. (% initial)
H_2O (initial)	—	802	37	1.0	0	100
Nitrate–1,10-phenanthroline	18 hr	372				
	24 hr	168		<0.1		
Dilute cobalt	10	200[a]	120[b]			
	15		320[b]			
Wash, distilled H_2O	10	200[a]	240[b]	<0.1	1.2	
Assay in chloride	30					85
Wash, distilled H_2O	10	200[a]	125[b]	<0.1	0.6	
Dilute cobalt	10	175	280[b]			
	25		384[b]			
	45		376[b]	<0.1	2.1	
Assay in chloride–dilute cobalt	30					120
Dilute cobalt	60					
Wash, distilled H_2O	15					
Dilute zinc–cobalt	12	1280[a]				
	25	1710[a]	188[b]		1.0	
	34	1830[a]	168[b]	2.6	0.8	
Assay in chloride–dilute cobalt	30					105
Wash, distilled H_2O	60	1680[a]	115[b]	2.3	0.5	

[a] Taken as 0.1-min count. [b] Taken as 0.25-min count.

curve b, is an alternative demonstration of the ability of the complex to enter and leave the lattice. This latter curve cannot be explained on the basis of a small amount of free zinc in equilibrium with the complex (complexes) in the external liquid and the assumption that the free zinc diffuses in while the complex remains outside. From the dissociation constants given by Kolthoff *et al.* (1951) the concentration of free zinc in the solutions used was much less than 10^{-5} M. If free zinc, in fact, had been the form diffusing into the crystal, the apparent diffusion constant would have been reduced from that equivalent to the curve a data by the concentration ratio or a factor greater than 1000. No such large reduction was observed.

The results of a series of assays on a single column of crystals exposed to a cobalt-containing solution are shown in Figure 4. A marked activation was noted in the cobalt-treated crystals. This effect was almost completely reversed by just washing the crystals with water. The further addition of zinc, box 7, had no detectable effect. As shown in box 8, the activation process was rapid. At least one-half of the maximum change was obtained in 5 min.

Table I gives the results of a sequence of combined activity measurements and metal content estimates made in a flow tube in the X-ray spectrometer. Although activation was clearly observed, the magnitude of the increase was never as large as that seen in Figure 4.

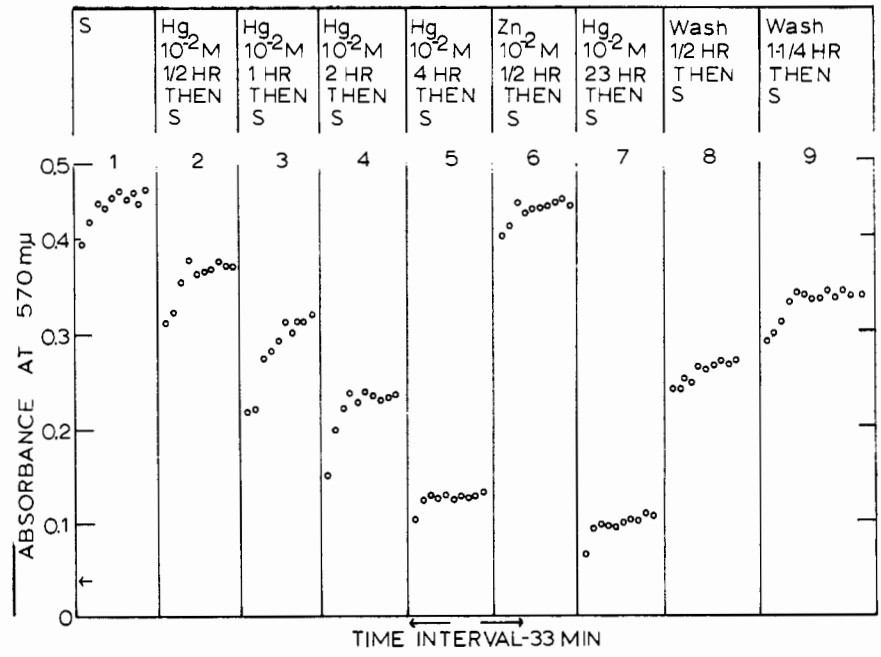

FIGURE 5: Inactivation of cross-linked carboxypeptidase crystals by mercury. The procedures are described in the legend to Figure 4. The mercury solution contained $HgCl_2$ 0.01 M, Tris 0.01 M, pH 7.5. At the end of this series the dry weight of the protein was found to be 0.47 mg.

In the presence of 1.0 M NaCl activation was observed without a large loss of zinc, and the cobalt content was readily decreased by washing with the substrate solution or just dilute buffer (sections 1 and 2 in Table I). When the cobalt treatment (sections 3 and 4 in Table I) was carried out in the presence of 0.5 M $NaNO_3$, the zinc content was very slowly lowered, a number of atoms of cobalt appeared to be bound per molecule of enzyme, and the activity remained above the starting level. Washing with a solution of zinc chloride at low ionic strength (section 5) removed all but about 1 atom of cobalt. The amount of zinc bound was much larger than 1 mole/mole of enzyme. This excess zinc was not removed by washing with distilled water and may be responsible for the inhibition seen in the assay. The extra zinc was removed with 1 M NaCl at slightly acid pH (section 6). At the end of the series of experiments the crystals appeared to contain one atom of zinc and about 0.5 atom of cobalt/enzyme molecule. The activity was above the starting value.

With the aim of characterizing the "zinc-free" cobalt enzyme in which there is no large excess of cobalt, the experiments shown in Table II were performed. A column of cross-linked crystals was treated with 1,10-phenanthroline in a sodium nitrate solution. After 24 hr of washing the zinc content was lowered to a barely detectable level. As shown in earlier experiments, the crystals then had little or no activity. Cobalt was readily taken up from a 10^{-4} M solution to give crystals with slightly more than one cobalt atom per protein molecule. These crystals were assayed in chloride and showed considerable activity but less than the starting value. A check of the metal content after the assay showed a loss of about one-half of the cobalt.

The cobalt content was restored to about 2 g-atoms/mole by further treatment with the cobalt solution. An assay then carried out in the presence of the same concentration of cobalt showed mild activation as previously observed. During all of these assays the zinc content was less than 10% of the starting value within statistical counting errors. The observed activity could not be attributed to the zinc, if both the activity and the zinc referred to bulk properties of the crystals.[1] A solution of zinc and cobalt, passed over the crystals, rapidly increased the zinc content to more than 2 g-atoms/mole of protein while at least 1 g-atom of cobalt appeared not to be displaced (third section from bottom of Table II). Assay again in the presence of cobalt showed the initial activity of the zinc protein. A final wash lowered the cobalt content, but not quite to the base line value. There appear to be a number of metal binding sites in addition to the major one at the active site.

The activity behavior of a column of cross-linked crystals in the presence of solutions of mercuric chloride is shown in Figure 5. There is a gradual loss of activity over a period of several hours, but the residual activity never goes to zero. The rate is much slower than the

[1] That this is a valid inference in shown by a separate series of experiments on the activity of zinc crystals extracted with 1,10-phenanthroline in 0.5 M $NaNO_3$. When the zinc content had been reduced to 55% of the starting value the measured activity was 60%. At a zinc level of less than 10% the activity was 8%.

TABLE III: Replacement of Zinc by Mercury.

Continuation of experiments with the column of crystals used for the data in Table II. Same general procedures with additional solutions. The cumulative time of exposure of the crystals to the exciting X-ray beam in the fluorescence measurements reported in this table was 6 min. The total time for this crystal batch was 17 min. Error estimates for columns 5 and 6 are ±0.1 g-atom/mole for Zn and ±0.5 g-atom/mole for Hg.

Assay in nitrate: carbobenzoxyglycylphenylalanine 0.02 M, $NaNO_3$ 1 M, sodium veronal 0.02 M, pH 7.50.
Nitrate–mercury dilute: $HgCl_2$ 10^{-4} N, $NaNO_3$ 0.5 M, Tris 0.01 N, pH 6.85
Nitrate–mercury: $HgCl_2$ 10^{-2} M, $NaNO_3$, 0.5 M, Tris 0.01 M, pH 5.20
Nitrate–Tris: $NaNO_3$ 0.5 M, Tris 0.01 M, pH 6.70
Nitrate–veronal: $NaNO_3$ 0.5 M, sodium veronal 0.02 M, pH 7.50.
Chloride–veronal: NaCl 1.0 M, sodium veronal 0.02 M, pH 7.50

Flowing Soln or Operation on Column of Crystals	Liquid Flow Time (per expt), min	Measured Counts at Peak Positions Including Background (cpm)		Estd Stoichiometry (g-atoms of metal bound/mole of protein)		Peptidase Act. (% initial)
		Zn Kα	Hg Lβ$_1$	Zn	Hg	
Flow tube						
H_2O	—	150	195			
0.01 M $HgCl_2$	—	—	501			
Nitrate–mercury dilute	15	920[a]				
	34	1000[a]		1.3		
	37		1030[a]		5.0	
Nitrate–mercury	12	460[a]				
	30	220[a]	2810[a]			
	45	170				
Wash, nitrate–Tris	4 hr	169[a]	1480[a]			
	17 hr	180[a]	1090[a]	<0.1	5.0	
Assay in nitrate	15					27
	18					35
	19					36
	21					40
	25					48
Wash, nitrate–Tris	1 hr	180[b]	870[a]	<0.1	4.0	
$ZnCl_2$ (0.01 M in nitrate–Tris)	16		690[a]			
	60		680[a]			
	75		650[a]			
Wash, nitrate–veronal	12	3010[a]				
	25	2500[a]				
	38	1830[a]				
	50	1380[a]	620[a]			
	71	1230[a]				
	85	1080[a]		1.4		
Assay in nitrate	15					140
Wash, nitrate–veronal	60	880[a]	625[b]	1.1	2.5	
Wash, chloride–veronal	30		440[a]			
	60		460[a]			
Wash, nitrate–veronal	20		490[b]			
Assay in nitrate	15					115
Wash, nitrate–veronal	15	795[b]	460[b]			
	16 hr	800[a]	475[b]	1.0	1.5	
Assay in chloride	15					115
Wash, nitrate–veronal	60	660[a]	460[a]	0.8	1.5	

[a] Taken as 0.1-min count. [b] Taken as 0.25-min count.

diffusion process and must represent either a very slow exchange or some other inherently slow-binding phenomenon. In the presence of added zinc, box 6, the activity is rapidly restored to the original level. In the last two boxes it is noted that in the absence of zinc, washing with just distilled water causes a gradual recovery of activity.

The primary object of the experiments shown in Table III was to prepare "zinc-free," mercury-containing crystals and to measure their activity. The mercuric nitrate was not completely soluble in the standard solvent containing veronal buffer at pH 7.5. Solutions of this metal salt in Tris buffer at somewhat lower pH values were thus used. The experiments shown in the table are discussed in sequence as performed. With nitrate as the principal anion and mercuric ion at 10^{-4} M, pH 6.8, substantial amounts of mercury were bound by the crystals without the displacement of the zinc. Note that the flowing solution, 10^{-4} M Hg, does not produce a count rate distinguishable from the background. The measured counts are thus entirely due to metal ions bound to the protein. When the mercury concentration was increased to 10^{-2} M (and the pH lowered to 5.2), the zinc was displaced. The replacement was apparently complete in less than 1 hr, presumably due to the presence of sodium nitrate as the main contributor to the ionic strength rather than the sodium chloride used in the column for the data in Figure 5. At this point an assay was carried out in the absence of any added metal ions. The initial activity was low but not zero. During the assay the activity appeared to increase, rising to almost 50% of the initial value after 30 min. The crystals still contained barely detectable amounts of zinc and about 4 atoms of mercury/molecule of enzyme. Exposure to a zinc solution, followed by washing, introduced somewhat more than one atom of zinc and removed about one atom of mercury. The standard assay now indicated 140% activity. Extensive further washing with both nitrate and chloride solutions reduced the zinc content to about 1 mole/mole of protein. The mercury content also dropped but was never reduced below about 1.5 moles/mole of protein. The activity of the crystals at this stage was slightly more than 100% and was the same whether measured in 1 M NaCl or 1 M $NaNO_3$.

Discussion

Vallee *et al.* (1960) reported that when native carboxypeptidase in solution was dialyzed against 2×10^{-3} M 1,10-phenanthroline, 1.0 M NaCl, 0.1 M Tris, pH 7.0, at 0°, the half-time for the loss of zinc from the dialysis bag was about 5 hr. In 20 hr the zinc content of the protein was less than 10% of the initial value. The data in Figure 1 show that crystals of the enzyme lose no detectable amounts of zinc after 20 hr of exposure to a five times higher concentration of the chelating agent at room temperature. This appears to be a real difference in the behavior of the solution and crystal forms of this protein. If the metal could move from the protein to the liquid-filled channels in the lattice, then it would be removed by washing, either as the free cation or the chelate complex. It was initially assumed that, whatever conformational change was required in the protein to permit removal of the metal ion, this change was prevented by the environment of the macromolecule in the crystal lattice. This conclusion subsequently had to be abandoned, however, when it was found that the metal was removed by 1,10-phenanthroline with a half time of about 2 hr in the presence of 0.5 M $NaNO_3$. Since neither very low nor high ionic strengths with chloride as the anion permit extraction of the metal, it must be assumed that the nitrate ion somehow affects the protein portion of the metalloenzyme. With the exception of such ions as perchlorate, the free zinc cation shows less tendency to associate with nitrate than with any other anion.

No explanation for this strange anion effect can be offered at this time. Since both the zinc enzyme and the apoenzyme prepared in solution and crystallized separately are isomorphous (Rupley and Neurath, 1960), there can be no great structural difference between these two forms. The process of metal removal might, of course, still involve substantial temporary structural changes. How such changes, if they occur, are affected by nitrate or chloride ions is obscure. The few comparative assays so far performed do not indicate any appreciable differences in catalytic activity of the enzyme in solutions containing chloride or nitrate ions.

From the data in Figure 4 and in Tables I and II, it is clear that activity does increase in the presence of dilute cobalt-containing solutions with the crystals as well as in solution (Coleman and Vallee, 1961). It should be remembered that the starting specific activity of the crystals is several hundredfold lower than that of the soluble enzyme (Quiocho and Richards, 1966). There is no reason to expect that relative activity changes for the two forms under a variety of conditions would necessarily bear any relation to each other. The observed activations have not been very reproducible. However, it is clear that in the presence of chloride ion cobalt is not able to displace the zinc. Cobalt is bound, however, and the observed activities are generally higher than the starting level. In the presence of nitrate very slow exchange of zinc for cobalt starts. Folk and Gladner (1960) have previously concluded from solution studies that cobalt can activate carboxypeptidase-A without replacing the atom of zinc.

By initial removal of zinc with 1,10-phenanthroline and subsequent addition of cobalt, crystals were prepared in which the active site (presumably) was occupied by cobalt. These crystals appeared to be about 20% more active than the initial zinc enzyme. The effect is probably real as the activity returned to the starting value when the cobalt was displaced by zinc.

The total amount of metal bound in many instances is greater than one mole per mole of protein. How many of these "extra sites" are an intrinsic property

of the protein molecules and how many are a peculiarity of the crystal lattice cannot be stated with any certainty. However, the enhanced or lowered activities observed with these extra sites occupied indicates that they are affecting the catalytic behavior of the protein. In the studies reported here, when more than one type of metal atom is bound to the protein, there is no way to tell which type is the principal occupant of the unique active site.

The influence of mercuric ions is perhaps even more striking. The very slow inactivation produced by 0.01 M $HgCl_2$ in 1.0 M NaCl is seen in Figure 5. The activity never reaches zero and is rapidly restored by added zinc. In the presence of nitrate as the principal anion (Table III), 10^{-4} M mercuric ion cannot displace the zinc but 5 moles of mercury/mole of protein are bound to other sites. When the mercury is 10^{-2} M, or perhaps because of the lower pH, the zinc is displaced. The activity of these mercury-containing crystals gradually increases during the assay until it is at least 50% that of the zinc enzyme. There are at least two possible explanations for this observation. (1) One might assume that the crystals used were sufficiently large that the turnover of the substrate was severely diffusion limited, and all activity measurements really reflected only the behavior of a thin surface layer. In addition, during the assay discussed above adventitious zinc was picked up in this layer but not in amounts, compared to the total crystal weight, to be detected by the fluorescence measurements. Thus the observed activity really referred to the zinc enzyme even though the bulk of the protein inside the crystals, were it susceptible to assay, would have been found inactive. (2) The mercury-containing enzyme in the crystals shows at least partial activity in the peptidase assay.

Although the crystals used were not accurately measured, visual inspection indicated that they were comparable to the ones labeled "medium" in the previous paper. Diffusion limitation was certainly present, but the authors do not believe it to have been severe enough to explain the results. They are inclined to believe the second explanation. In experiments with columns of crystals where the zinc had been removed, activities, measured with the same substrate solutions, were zero or very low and showed no tendency to increase with time. Thus there was no evidence for pickup of trace amounts of contaminating zinc which might have survived the reagent purification procedure.

Reinsertion of zinc in stoichiometric amounts into the "mercury enzyme" still leaves 2 to 3 atoms of mercury/mole of enzyme. This complex has even higher activity than the starting zinc enzyme. No amount of washing in any of the salt solutions tried has been able to reduce the level of this very firmly bound mercury below 1–2 g-atoms/molecule of protein. The activity remains somewhat higher than the starting level.

Toward the end of the two series of experiments reported in Figure 5 and Table III, the two different crystal batches both contain zinc, presumably at the active site, and mercury atoms bound elsewhere. In the first case there is inhibition of peptidase activity which can be reversed, at least in part, by washing, in the second there is activation. The history of the two batches is not the same; in particular, the solvents used had different ionic composition. Since no metal analyses were made on the actual batch used in Figure 5 no detailed comparison is warranted. Together, however, the two sets of experiments strongly support the existence of multiple metal binding sites in addition to the one at the active center.

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