

Binding of Divalent Copper Ions to Aspartic Acid Residue 52 in Hen Egg-white Lysozyme

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(Received 13 September 1973, and in revised form 7 February 1974)

Divalent copper was found to inhibit non-competitively the lysis of *Micrococcus lysodeikticus* cells by hen egg-white lysozyme, with an inhibition constant $K_a = 3.8 \times 10^2 \text{ M}^{-1}$. The association constants of Cu^{2+} for lysozyme and for a derivative of lysozyme in which tryptophan residue 108 was selectively modified, were measured spectrofluorimetrically and found to be $1.8 \times 10^2 \text{ M}^{-1}$ and $1.0 \times 10^3 \text{ M}^{-1}$, respectively. The electron spin resonance spectrum of Cu^{2+} was not affected by the addition of lysozyme, whereas many new lines appeared on addition of the modified protein. This was interpreted as evidence for the binding of Cu^{2+} in the neighbourhood of tryptophan 108. To unequivocally establish the site of ligation of Cu^{2+} , crystals of lysozyme soaked in Cu^{2+} were examined by X-ray crystallography and the results compared to those obtained from crystals of native lysozyme. Cu^{2+} was found to be located 2 to 3 Å from the carboxyl side-chain of aspartic acid 52, 5 Å from the carboxyl of glutamic acid 35 and about 7 Å from tryptophan 108.

The addition of a saccharide inhibitor to lysozyme was found to increase the association constant of Cu^{2+} for lysozyme from a value of $1.8 \times 10^2 \text{ M}^{-1}$ to $6.0 \times 10^2 \text{ M}^{-1}$. This finding was interpreted as indicative of a change in conformation around tryptophan 108 and glutamic acid 35 induced by the interaction of saccharides with the enzyme, which affects the metal binding properties of aspartic acid 52.

1. Introduction

Divalent cations such as cobalt (Sakura & Rupley, unpublished data and personal communication, 1972), manganese (Gallo *et al.*, 1971), and copper (Feeney *et al.*, 1956) inhibit the enzymic activity of hen egg-white lysozyme, although only at relatively high concentrations (10^{-3} to 10^{-2} M). Until now, no experimental information on the nature of the interactions between such cations and the enzyme has been presented. Magnetic resonance studies of lysozyme complexes with Co^{2+} (McDonald & Phillips, 1969) and with Mn^{2+} (Gallo *et al.*, 1971) have led to the suggestion that these ions inhibit the enzyme activity by binding between the residues Glu-35 and Asp-52, the catalytic groups in its active site (Phillips, 1967).

We have investigated the binding of Cu^{2+} to lysozyme using enzymic and spectroscopic (fluorimetry and electron spin resonance) techniques. In some of the experiments, we also measured the binding of Cu^{2+} to a lysozyme derivative in which Trp-108 was specifically modified by iodine oxidation (Hartdegen & Rupley, 1964; Teichberg, 1972). To establish unequivocally the site of ligation of Cu^{2+} in the

lysozyme molecule, a study of Cu^{2+} -bound lysozyme crystals was carried out by X-ray crystallography. The electron density difference maps of lysozyme *versus* Cu^{2+} -lysozyme clearly showed a number of peaks, one of which was within 2 to 3 Å of one of the carboxyl oxygens of Asp-52. Additional peaks were also detected, but they were outside the active site of the enzyme.

2. Materials and Methods

(a) *Materials*

Hen egg-white lysozyme (twice recrystallized, salt-free) was a product of Worthington Biochemicals. Lysozyme, oxidized specifically at Trp-108, was prepared by the method of Hartdegen (1967), and lyophilized. $(\text{GlcNAc})_4$ was prepared by the method of Rupley (1964) from a partial hydrolysate of chitin and further purified by paper chromatography on Whatman no. 3 paper, using as solvent butanol/acetic acid/water (25:6:25, upper phase). The cell wall tetrasaccharide $(\text{GlcNAc-MurNAc})_2$ was isolated from *Micrococcus lysodeikticus* as described previously (Sharon, 1967).

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was an Analar product of British Drug Houses. MES†, A grade, was from Calbiochem. All other chemicals were of the highest purity commercially available, and twice-distilled water was always used.

(b) *Methods*

(i) *Enzymic assay*

Lysozyme activity was estimated spectrophotometrically by following the rate of lysis of *M. lysodeikticus* cells, using the method of Shugar (1952). The decrease in turbidity of a cell suspension was measured at room temperature as a function of time at a wavelength of 450 nm with a Beckman DB spectrophotometer. The reciprocal initial rate $1/v$ was calculated from the equation $1/v = 0.43 t / (\log A_{t=0} - \log A_t)$ where $A_{t=0}$ is the absorbance of the cell suspension at time $t = 0$ and A_t is its absorbance after 1 min ($t = 1$). The concentration of lysozyme was estimated by optical density measurement using an extinction coefficient of 26.0 at 280 nm for a 1% by weight protein solution in water (Bradshaw *et al.*, 1967). Cu^{2+} concentration was estimated by weighing the required quantity of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, dissolution in buffer and dilution to an appropriate volume.

(ii) *Fluorescence measurements*

Fluorescence measurements were made with an Aminco-Bowman spectrofluorimeter equipped with a Rikadenki X-Y recorder. A Hanovia 901 C-150 W Xenon lamp was used for excitation at 285 nm. The emission was measured using a 1P-28 photomultiplier tube. All fluorescence measurements were performed in 1-cm quartz cuvettes (manufactured by Hellma) kept at 23°C in a thermostatically controlled cell jacket. Solutions were passed through Millipore filters (HAWP 0.45) using a Swinnex-13 Millipore adaptor. Dilutions were made to obtain a final absorbance of less than 0.1 in a 1-cm path length cell at 285 nm.

Association constants were determined by fluorimetric titrations at 340 nm as described by Chipman *et al.* (1967). Solutions of lysozyme, CuSO_4 and buffer, were mixed in the fluorescence cell (0.1 ml of a 0.4 mg/ml protein solution, 0.005 ml to 0.100 ml of 2.5×10^{-2} M- CuSO_4 and 0.1 M-MES buffer to 1 ml) and their fluorescence intensities, relative to that of reference solutions containing only the protein and buffer, were determined. The absorption at 285 nm and the fluorescence at 340 nm of solutions of CuSO_4 alone in MES buffer were also measured and were used to correct the observed fluorescence of Cu^{2+} -lysozyme (correcting factor = $\text{antilog } \frac{1}{2} \text{ O.D.}_{285}$). At the highest Cu^{2+} concentration used, such corrections were less than 10% of the total change $[F_0 - F]$ in the lysozyme fluorescence, where F_0 is the fluorescence of the protein alone and F is its fluorescence in the presence of a given concentration of Cu^{2+} .

† Abbreviation used: MES, 2-(*N*-morpholino)ethane-sulphonic acid.

(iii) *Electron spin resonance*

Electron spin resonance spectra were recorded at room temperature with a Varian E3 spectrometer equipped with a flat aqueous sample cell.

 (iv) *X-ray crystallography*

The X-ray crystallographic studies were performed essentially as described by Moulton *et al.* (1973). Tetragonal crystals of lysozyme (Alderton & Fevold, 1946) were grown at pH 4.7 in 0.02 M-acetate buffer, containing 5% NaCl. Individual crystals (space group $P4_32_12$, $a = b = 79.1 \text{ \AA}$, $c = 37.9 \text{ \AA}$), about 0.7 mm in the longest dimension, were transferred to 1.5-mm diameter quartz capillaries containing about 0.15 ml mother liquor. About 2 mg of solid $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ were added to the mother liquor to obtain a ratio of about 1:50 lysozyme to CuSO_4 (molar ratio).

The crystals were then allowed to soak at room temperature for 2 days and the excess mother liquor removed from the tubes with a syringe and filter paper. Precession photographs with $\mu = 18^\circ$ (minimum spacing 2.5 \AA) were taken of the hko , $0kl$ and hhl zones. These zones are centrosymmetric for this space group ($P4_32_12$). Photographs were taken with 2 films in a pack and exposures of 30 to 40 h, using a copper X-ray tube running at 40 kV, 28 mA with a nickel filter. No crystal was exposed for more than 50 h. The crystals were isomorphous with those of native lysozyme and showed only small changes in intensities. The complete diffraction patterns were recorded on magnetic tape, using an Optronics photoscanner, and the integrated intensities of the reflections, corrected for backgrounds, were obtained by processing on an IBM 370/165 computer. Intensities within a pack were scaled together, corrected for Lorenz and polarization factors, and scaled to those of the native crystals. Difference Fourier projections were calculated, using as amplitudes the differences between the amplitudes of native lysozyme (Phillips, personal communication) and of Cu^{2+} -lysozyme, weighted with the native figures of merit, and native phases.

3. Results

Figure 1 presents the results of measurements of the enzymic activity of lysozyme in the presence of increasing concentrations of Cu^{2+} in the co-ordinate system of Dixon (1953). The lines, representing assay mixtures with two different substrate concentrations, intersect on the abscissa at a single point indicating the non-competitive nature of the inhibition of lysozyme activity by Cu^{2+} ions. A value of $K_i = 2.6 \times 10^{-3} \text{ M}$ ($K_{\text{Cu}^{2+}} = 3.8 \times 10^2 \text{ M}^{-1}$) can be derived from the position of the

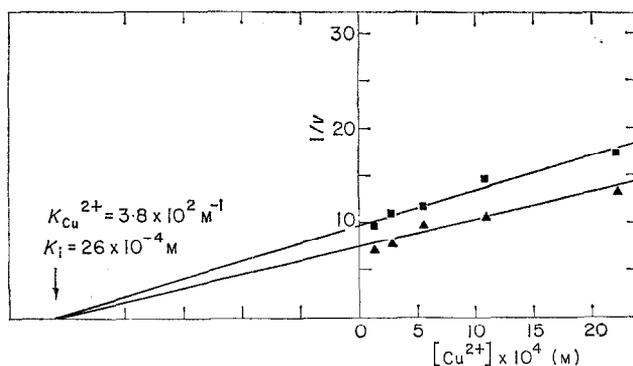


FIG. 1. Non-competitive inhibition of lysozyme by Cu^{2+} ions. The enzymic assay was carried out using *M. lysodeikticus* dried cells as substrate (at concentrations of 93 (■) and 116 (▲) $\mu\text{g/ml}$) at pH 5.0 in a non-complexing buffer of 0.1 M-MES and at room temperature. Enzyme concentration was $3.45 \times 10^{-7} \text{ mol/l}$. The concentration of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ varied from $1.1 \times 10^{-4} \text{ mol/l}$ to $2.2 \times 10^{-3} \text{ mol/l}$.

intersection of the lines on the abscissa. The inhibition of lysozyme by Cu^{2+} in the range of cation concentrations used was completely reversed by an equimolar amount of EDTA.

The effect of the Cu^{2+} ion on lysozyme was further studied by fluorescence spectroscopy. At a concentration of 2×10^{-3} mol/l, Cu^{2+} was found to produce a 20% quenching of the fluorescence of both lysozyme and lysozyme-saccharide complexes (lysozyme concn, 2.75×10^{-6} mol/l) but did not produce any shift in the emission wavelength maximum.

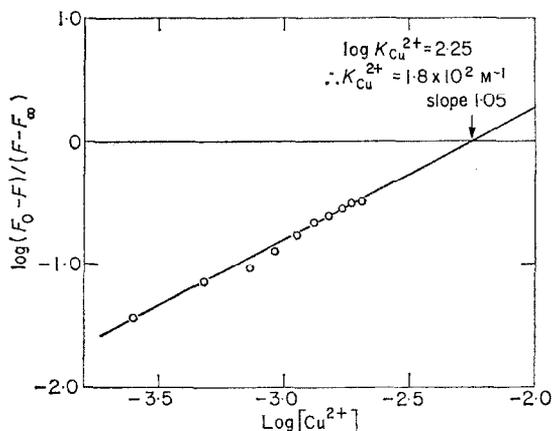


Fig. 2. Spectrofluorimetric determination of the association constant of Cu^{2+} for lysozyme at pH 5.0 (0.1 M-MES buffer) and 25°C. See details in text.

Figure 2 represents the results of a spectrofluorimetric determination of the association constant of Cu^{2+} for lysozyme. $\text{Log}[(F_0 - F)/(F - F_\infty)]$ is here plotted against log Cu^{2+} , where F_0 , F and F_∞ † are the fluorescence intensities of solutions at 340 nm of enzyme alone, enzyme in the presence of given concentration of Cu^{2+} and enzyme saturated with Cu^{2+} , respectively. The slope of the line is unity, indicating formation of a one-to-one complex. The association constant of Cu^{2+} to lysozyme in the presence of saturating amounts of $(\text{GlcNAc})_4$ measured as above, was found to be three times higher than the association constant of the metal to the free enzyme (Table 1). For comparison, we also measured the association constant of $(\text{GlcNAc})_4$ to lysozyme in MES buffer and found a value of $1.1 \times 10^5 \text{ M}^{-1}$, which is very close to that found in other buffers (Chipman *et al.*, 1967). We were, however, unable to measure the association constant of $(\text{GlcNAc})_4$ to lysozyme in the presence of a saturating amount of Cu^{2+} because of the poor solubility of Cu^{2+} at the high concentration needed for the saturation of the enzyme. In addition to quenching the fluorescence of lysozyme, Cu^{2+} was found to quench the fluorescence of iodine-oxidized lysozyme and of its complex with $(\text{GlcNAc})_4$, making it possible to measure the binding constant of Cu^{2+} in these systems too (Table 1). The results show that Cu^{2+} binds to both iodine-oxidized lysozyme and its saccharide complex with an identical strength and in a one-to-one ratio.

† The latter value is obtained by extrapolation, as previously described (Chipman *et al.*, 1967).

TABLE I

Association constants of Cu^{2+} to lysozyme, iodine-oxidized lysozyme and their complexes with saccharides

System†	K_a (M^{-1})
Lysozyme	1.8×10^{12} §
Lysozyme + 2.5×10^{-3} M-(GlcNAc) ₄ ‡	6.0×10^2
Iodine-oxidized lysozyme	1.0×10^3
Iodine-oxidized lysozyme + 2.5×10^{-3} M-(GlcNAc) ₄	1.0×10^3
Iodine-oxidized lysozyme + 10^{-3} M-(GlcNAc-MurNAc) ₂	1.0×10^3

† All association constants were measured spectrofluorimetrically at room temperature. All solutions were in 0.1 M-MES at pH 5.0. The protein was at a concentration of 2.75×10^{-6} mol/l. Saccharides do not absorb or fluoresce between 280 and 440 nm. The fluorescence of the protein was followed at 340 nm.

‡ Increasing amounts of Cu^{2+} were added to a lysozyme solution (2.75×10^{-6} mol/l in 0.1 M-MES) in the presence of saturating amounts of (GlcNAc)₄ (2.5×10^{-3} mol/l), which corresponds to a saturation of 99.5% of the enzyme on the basis of the $K_a = 1.1 \times 10^3 \text{ M}^{-1}$ for this saccharide under the above conditions.

§ A value of $3.8 \times 10^2 \text{ M}^{-1}$ was found by inhibition studies (see Fig. 1).

The interaction of Cu^{2+} with lysozyme and with iodine-oxidized lysozyme was examined by electron spin resonance. Addition of lysozyme to a solution of Cu^{2+} caused a decrease in the intensity of the electron spin resonance signal, but did not change its shape (Fig. 3, curves A and B). However, the electron spin resonance spectrum of Cu^{2+} was dramatically affected by adding equimolar amounts of the iodine-oxidized enzyme, with many new lines appearing (Fig. 3, curve C).

Figure 4 shows three projections of the difference in electron density between Cu^{2+} -lysozyme and native lysozyme. The peaks correspond to three main locations in the lysozyme molecule, for each of which there are several crystallographically equivalent peaks related by 4-fold or 2-fold symmetry axis. In one case (peak C in $hk0$ projection) two equivalent peaks are projected on top of each other. Because of this, peak C appears to have twice its true height in the $hk0$ projection.

The fractional co-ordinates observed for the three main peaks are:

	x	y	z
A	0.11	0.30	0.58
B	-0.20	0.20	0.25
C	-0.12	0.12	0.38

Distances between the locations of these peaks and all atoms of the lysozyme molecule were calculated.

Peak A is 3.2 Å from $\text{O}^{\delta 2}$ and 1.5 Å from $\text{O}^{\delta 1}$ of Asp-52. This short Cu^{2+} — $\text{O}^{\delta 1}$ distance would be energetically unfavorable. So, the insertion of the Cu^{2+} ion probably causes some slight distortion of the Asp-52 side chain. However, this might not be observable in an electron density projection, and, in fact, the approximate 6 Å diameter of peak A is large enough to include electron density changes due to such a small shift of the carboxyl side chain of Asp-52.

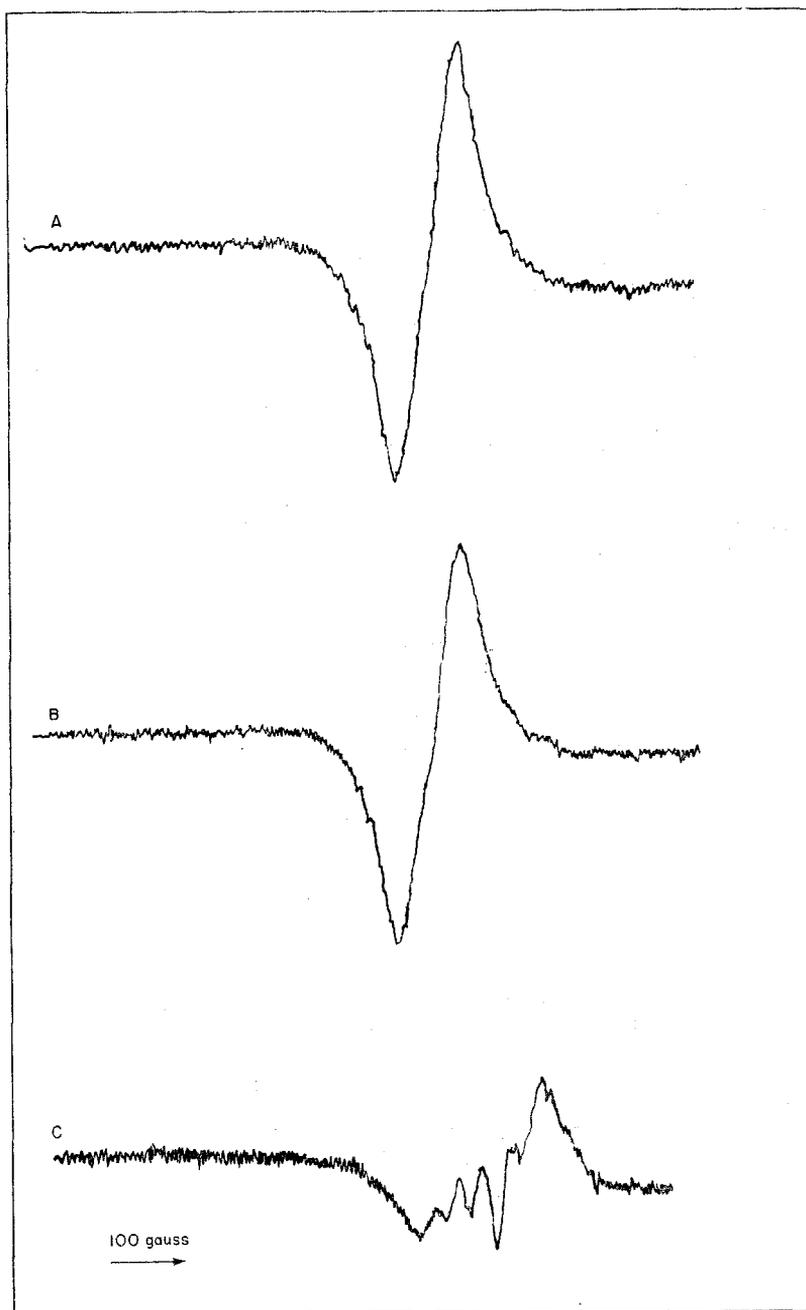


FIG. 3. Electron spin resonance spectrum of 5×10^{-4} M-Cu²⁺ in 0.1 M-NaCl, pH 7.0 (curve A); curve B: the same plus 5×10^{-4} M-lysozyme; curve C: the same plus 5×10^{-4} M-iodine-oxidized lysozyme, measured with a Varian E3 spectrometer. Field set 3080 gauss; scan range 1000 gauss; modulation amplitude 12.5 gauss; receiver gain 1.25×10^5 ; microwave power 125 mW.

Peak B lies on the 2-fold axis, and is close to the carboxyl end of the lysozyme molecule (Leu-129) and $\text{N}\zeta$ of Lys-13. In this location it can make an intermolecular bridge, connecting the Leu-129 carboxyl groups of adjacent, symmetry related, lysozyme molecules. The bridge probably includes hydrogen bonds between COO^- and the hydrated water of the Cu^{2+} ion.

Peak C is found to be close to $\text{N}\eta^1$ of Arg-14. As it is the weakest peak, we are not certain whether it represents a significant binding site.

The difference maps also contain holes. The distances between these and the protein molecule were calculated. Except for one case, where a hole lies near the carbonyl group of Arg-128, none of these holes was found to be closer than 5.0 Å to the protein, which suggests that there are no marked conformational changes in the protein itself upon binding to Cu^{2+} .

4. Discussion

The active site of lysozyme is composed of six subsites, *A-F*, in which a hexasaccharide substrate can bind (Phillips, 1966; Chipman & Sharon, 1969). The carboxyl groups of Asp-52 and Glu-35 are situated between subsites *D* and *E* on both sides of the glycosidic bond to be split and are believed to be the catalytic groups of the enzyme. The non-competitive character of the inhibition of lysozyme by Cu^{2+} therefore excludes the possibility that the Cu^{2+} ion is ligated to both Asp-52 and Glu-35, as previously suggested (McDonald & Phillips, 1969; Gallo *et al.*, 1971). The association constant of Cu^{2+} to lysozyme measured by spectrofluorimetry was found to be $K_a = 1.8 \times 10^2 \text{ M}^{-1}$ and by inhibition studies $K_a = 3.8 \times 10^2 \text{ M}^{-1}$. In order to obtain an additional estimate of this constant, binding measurements by equilibrium dialysis were attempted. Since radioactive $^{64}\text{Cu}^{2+}$ has a short lifetime ($t_{1/2} = 12.8 \text{ h}$), we used non-radioactive Cu^{2+} , which was estimated colorimetrically by the method of Felsenfeld (1960). However, it was difficult to estimate accurately the small difference in Cu^{2+} concentration between the "outer" and "inner" cells (i.e. with and without protein). We could, nevertheless, obtain an approximate value for the association in the range from 1.0 to $9 \times 10^2 \text{ M}^{-1}$. In view of the difficulty in estimating weak association constants accurately, such as those found in the present work, the values for the Cu^{2+} -lysozyme system by the three different methods used may be considered as being in good agreement.

The three tryptophan residues in the active site of lysozyme are the main contributors to the fluorescence emitted by the enzyme (Lehrer & Fasman, 1967; Teichberg, 1972). Since Cu^{2+} quenches the fluorescence of lysozyme, presumably by an electron transfer mechanism (Luk, 1971), this is an indication of the relative proximity of the Cu^{2+} site to fluorescent chromophores. Ni^{2+} , which, in comparison to Cu^{2+} , is a weaker inhibitor of lysozyme, was not found to quench the fluorescence of lysozyme at concentrations of up to $2 \times 10^{-3} \text{ M}$, whereas Cu^{2+} at the same concentration produced a 20% quenching. This indicates that quenching of lysozyme fluorescence results from the occupation of a site in the enzyme and not from the mere presence of metals in the bathing solution. The fact that Cu^{2+} quenches the fluorescence of iodine-oxidized lysozyme (in which the main fluorescent chromophore Trp-108 is oxidized into an oxindole moiety (Teichberg & Sharon, 1970)), indicates that the metal ion affects the fluorescence emitted by other tryptophans in the enzyme molecule. However, from the electron spin resonance data on

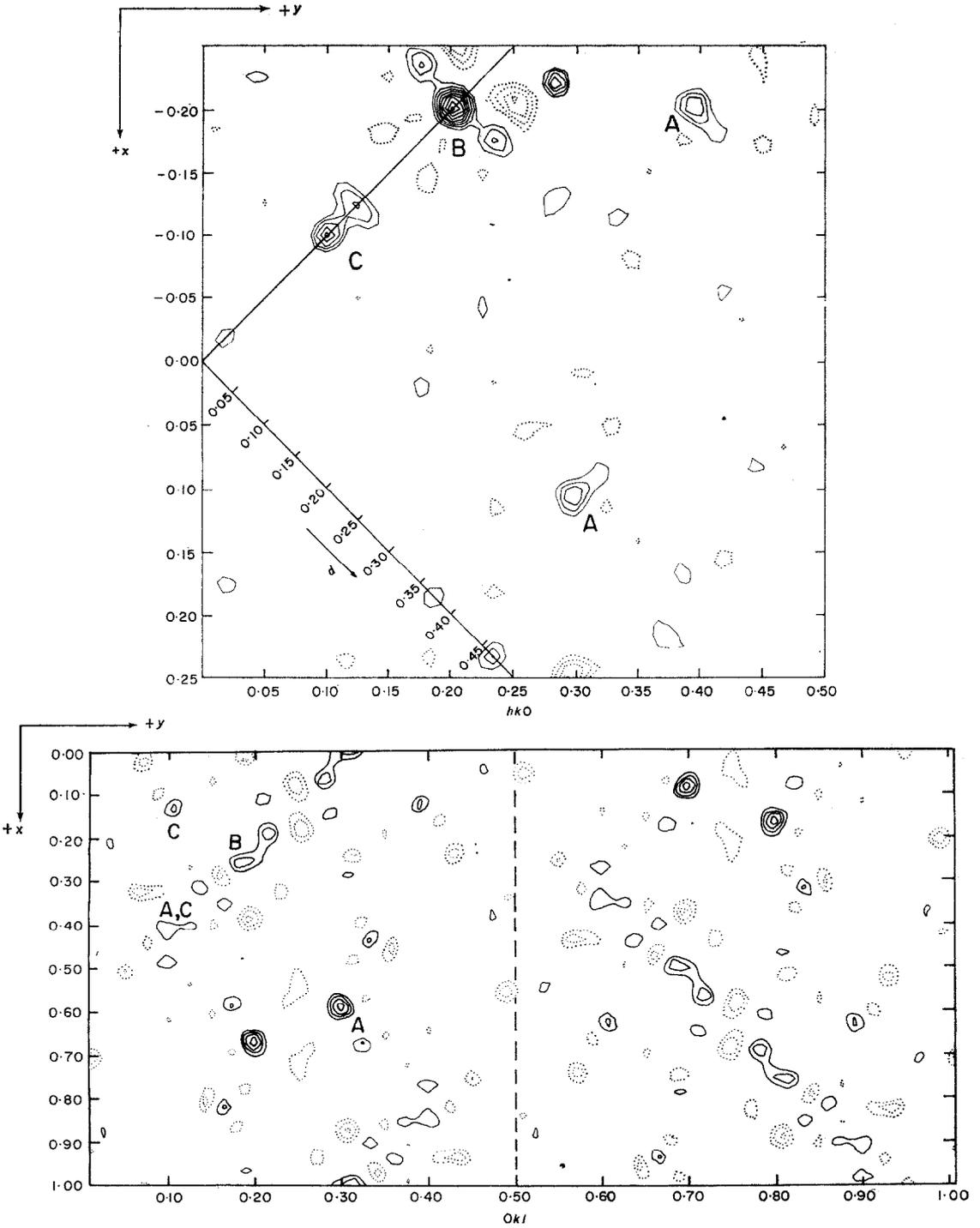


FIG. 4.

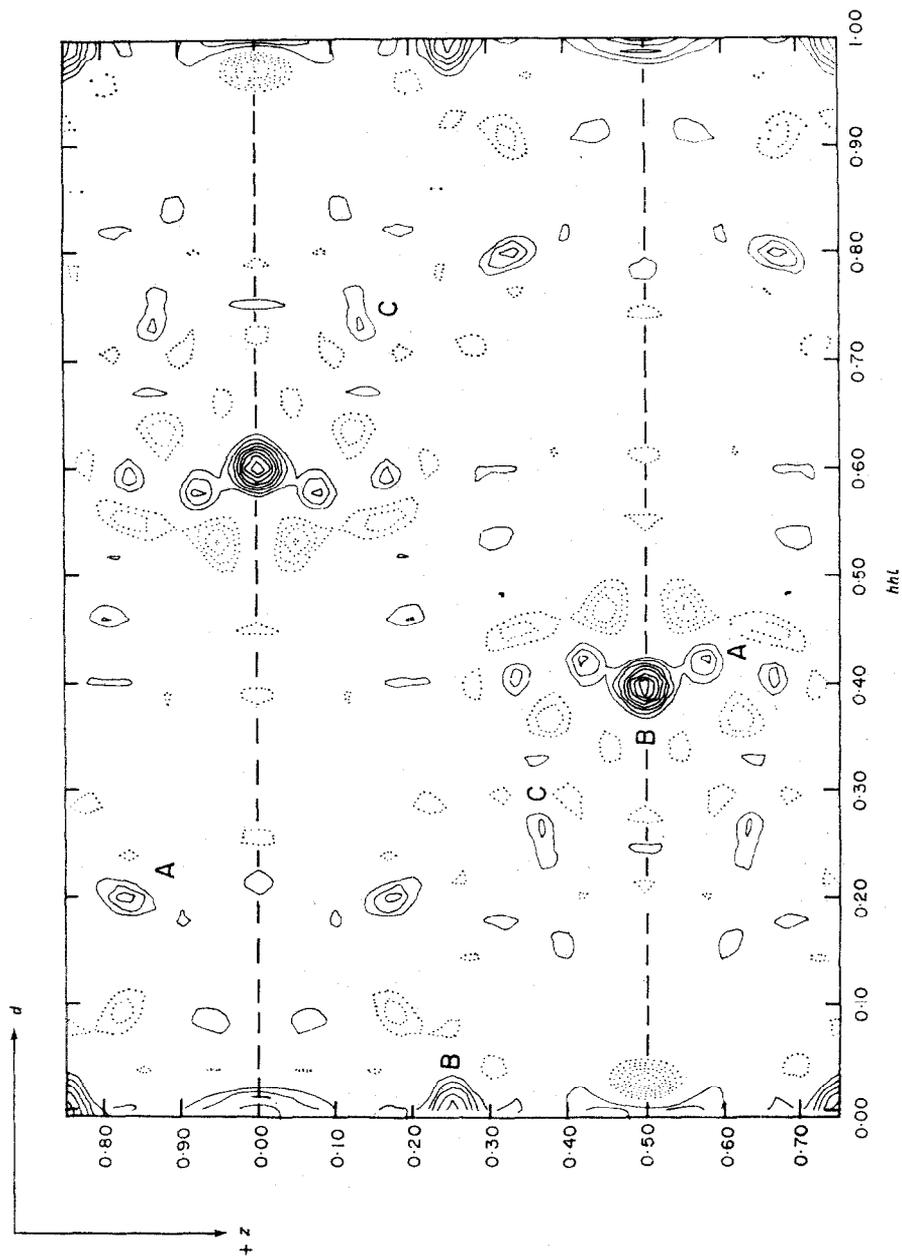


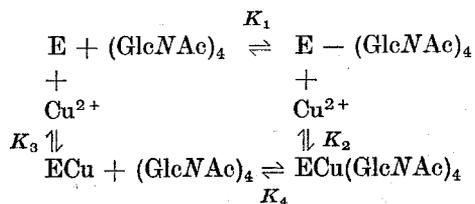
FIG. 4. Difference Fourier projection maps of the complex of tetragonal egg-white lysozyme with Cu^{2+} . The co-ordinates of the positive peaks are given in the text. Symmetry related peaks that correspond to equivalent positions in the different molecules within the unit cell are labelled with the same letter.

the Cu^{2+} complexes of lysozyme and of iodine-oxidized lysozyme, a clearer conclusion as to the location of the Cu^{2+} can be drawn. As can be seen from Figure 3, modification of Trp-108 results in marked changes in the electron spin resonance spectrum of the Cu^{2+} bound to the protein. It was therefore suggested (Teichberg, 1972) that the Cu^{2+} binds near Trp-108, most likely ligating the nearby Glu-35. This suggestion seemed especially appealing to us since there is evidence (Bedell, 1971) that the modification of Trp-108 by iodine oxidation also affects the relative position of Glu-35 in the protein.

To obtain further evidence on the location of the Cu^{2+} bound to lysozyme, we have examined by X-ray crystallography, the structure of a Cu^{2+} -lysozyme complex, in comparison to that of lysozyme alone. The results show the presence of an electron density peak, in direct contact with the carboxyl side-chain of Asp-52, and not as anticipated, very close to Glu-35. The presence in the difference Fourier maps of electron-density peaks other than that of Cu^{2+} bound to Asp-52, is due to copper ions bound outside the active site of the enzyme, between two molecules in the crystal. Obviously, such binding could not have been detected by us in solution, since the methods used (inhibition of enzyme action and spectrofluorimetry) measure only interactions at the active site of the enzyme.

Assuming that binding in solution is the same as that observed in the crystal, the inhibitory effect of Cu^{2+} on the activity of lysozyme must be ascribed to its interaction with Asp-52, one of the carboxyl groups believed to be involved in the catalytic action of the enzyme. It is possible that other divalent metal ions, such as Co^{2+} , Mn^{2+} and Ni^{2+} , which bind to lysozyme two to three times more weakly than Cu^{2+} (Ikeda & Hamaguchi, 1973), also interact with the enzyme through the carboxyl of Asp-52, and not through that of Glu-35. If this is so, then the effect of these metal ions on the spectral properties of the enzyme, originally ascribed to interaction with Glu-35 (Ikeda & Hamaguchi, 1973), may be the result of an indirect effect on the environment and pK of the neighbouring Asp-52. That the carboxyl group of Asp-52 affects the properties of the carboxyl of Glu-35 is well established (Parsons & Raftery, 1972; Sharon & Eshdat, 1974).

The non-competitive character of the lysozyme inhibition by Cu^{2+} allows us to interpret our results by the following mechanism, which has also been proposed very recently and independently by Ikeda & Hamaguchi (1973):



Since Cu^{2+} binds to Asp-52, it inhibits lysozyme activity. When a saccharide such as $(\text{GlcNAc})_4$ is bound to the enzyme (in subsites *A-B-C*) a change in conformation takes place by which Glu-35 moves closer to Trp-108 (Teichberg & Shinitzky, 1973). Such movement changes the properties of the nearby side-chains of Asp-52 and facilitates the binding of Cu^{2+} , which is now more strongly associated to the lysozyme-saccharide complex than it is to free lysozyme ($K_3 = 1.8 \times 10^2 \text{ M}^{-1}$, $K_2 = 6.0 \times 10^2 \text{ M}^{-1}$). Interestingly, stronger binding of metal ions to lysozyme-saccharide

complexes than to the enzyme alone, was also reported by Ikeda & Hamaguchi (1973).

In the cyclic mechanism presented above, the law of microscopic reversibility leads to the relation $K_1K_2 = K_3K_4$. We can, therefore, calculate $K_4 = (K_1K_2)/K_3$ from the known values of $K_1 = 1.1 \times 10^5 \text{ M}^{-1}$, K_2 and K_3 . Such a calculation gives a value of $K_4 = 3.6 \times 10^5 \text{ M}^{-1}$. The presence of Cu^{2+} should thus enhance the binding of saccharides to lysozyme. Again, this is in agreement with the results of Ikeda & Hamaguchi (1973) who found that the binding constant of $(\text{GlcNAc})_3$ with lysozyme in the presence of Mn^{2+} was about twice as large as the constant in the absence of Mn^{2+} .

The binding of Cu^{2+} to iodine-oxidized lysozyme is stronger than its binding to lysozyme- $(\text{GlcNAc})_4$, possibly because the modification of Trp-108 has brought a larger displacement of Glu-35 (2 Å, Bedell & Blake, 1970) and a concomitant change in the properties of the side-chain of Asp-52, than that resulting from the formation of the enzyme-saccharide complex. An identical value of K_a is found for the binding of Cu^{2+} to iodine-oxidized lysozyme and to its complex with $(\text{GlcNAc})_4$ and we, therefore, assume that in this case the modified Trp-108 does not move with respect to Glu-35 and Asp-52 as a result of saccharide binding.

We thank Professor D. C. Phillips for providing us with the structure factors of hen egg-white lysozyme, and Dr Gerson H. Cohen for his computer program for X-ray photograph scanning and data processing. Thanks are also due to Dr C. Nicolau and Dr Y. Eshdat for their help in some of the experiments and to Professor W. Traub for helpful discussions.

This work was supported in part by grant no. GM19143 from the National Institutes of Health, United States Public Health Service.

One of us (J. M.) wishes to thank the European Molecular Biology Organization for a research fellowship (1970 to 1972).

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