THE DISULFIDE BOND IN CARBOXYPEPTIDASE A: A CHLORIDE ION NMR STUDY

Robert G. Bryant*, Department of Chemistry, Stanford University, Stanford, Calif.; Herman J. C. Yeh and Thomas R. Stengle, Department of Chemistry, University of Massachusetts, Amherst, Mass.

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SUMMARY

Carboxypeptidase A was assayed for active sulfhydryl groups by titration with $HgCl_2$. A chloride ion probe NMR technique was utilized to observe the behavior of the mercuric ion. Although mercuric ion was found to bind to the enzyme, it could be removed by dialysis showing that the binding cannot be through a sulfur atom. The absence of sulfhydryl groups indicates that the two half cystine residues in the molecule must be linked by a disulfide bond.

The enzyme carboxypeptidase A is reported to contain two half cystine residues, however the nature of these groups in the native enzyme has been the subject of investigation for some time. Earlier workers suggested that the sulfur was present as free sulfhydryl (1,2), while recent x-ray evidence indicates the existence of a disulfide bridge (3). However, there is no published chemical evidence for either case. Recent work has demonstrated that the NMR halogen ion probe technique is particularly well suited for investigating protein sulfhydryl groups (4,5). This note describes chloride ion probe measurements which provide strong evidence for the absence of free sulfhydryl groups in the enzyme.

A protein sulfhydryl group can be detected by titrating

*Present address: Department of Chemistry, University of Minnesota, Minneapolis, Minn.

the protein with mercuric chloride in the presence of sodium chloride and observing the behavior of the ^{35}Cl NMR line width. Because of the very high affinity of mercury for sulfur, mercuric ions bind to sulfhydryl groups quantitatively even in acidic solutions high in chloride ion concentration. The rapid exchange of chloride ions between the solvent and the sulfhydryl bound mercury species results in a composite NMR line, the width of which is the weighted average of the line widths associated with each chloride ion environment. Because the line width at each site is proportional to the correlation time for the reorientation of the electric field gradient at the quadrupole relaxed chlorine nucleus, the relatively slow motion of the protein sites results in a large contribution to the total line width, making the NMR spectrum a sensitive measure of the protein bound mercury concentration. When protein binding sites are saturated with mercury, further addition of metal results in relatively small line width increases because of the rapid motion of the $HgCl_4^=$ species compared to that of the protein. Thus ^{35}Cl NMR provides a simple and direct method for studying the binding of mercury to proteins, particlarly at sulfhydryl groups.

METHODS AND MATERIALS

The NMR measurements were made on Varian spectrometers at 4.33 and 5.8 MHz employing techniques described previously (5). Carboxypeptidase A was obtained from Worthington Biochemical Corporation. Samples were prepared by dialysis of the enzyme suspension against a phosphate buffer in 1.00 M sodium chloride; enzyme concentrations were determined spectrophotometrically at 278 mμ. The apo enzyme solutions were prepared by the method of Vallee, et al. (6). Metal titrations were carried out by

adding microliter quantities of 2.00×10^{-2} M mercuric chloride in buffer solution to the enzyme samples.

RESULTS AND DISCUSSION

The ^{35}Cl NMR line width was used to follow the addition of mercuric ion to both the native and apo carboxypeptidase samples. The results are summarized in Figure 1. In both cases an increase in chloride line width was observed due to binding of mercury to the enzyme. The small slope and the absence of a distinct break in the titration curve are inconsistent with the presence of an accessible sulfhydryl group in the enzyme. This conclusion was checked by dialysis of the most concentrated mercury solution against 1.00 M sodium chloride and buffer for 1½ hours which resulted in complete removal of all metal added.

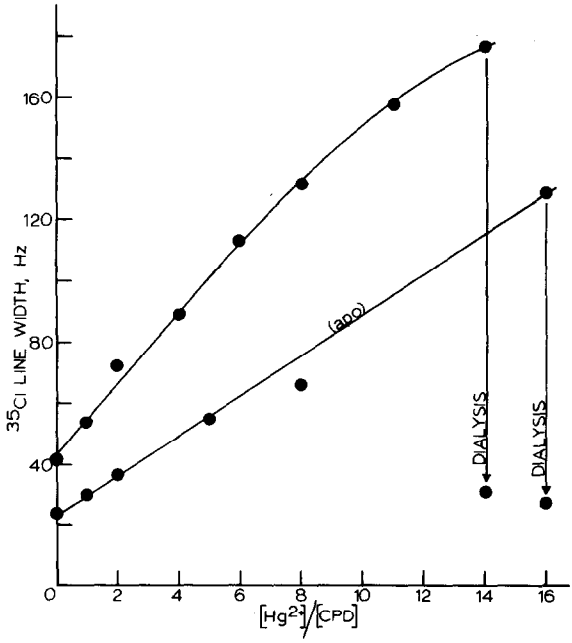

Figure 1. ^{35}Cl NMR line width as a function of mercury concentration for 1.00 M NaCl solutions containing 1.0×10^{-4} M carboxypeptidase at pH 7.0. At the end of each titration, the resulting solution was dialyzed against NaCl and buffer; the removal of all mercury is indicated by the decrease in line width to the initial value.

In control experiments using the protein bovine serum mercaptalbumin, identical dialysis did not remove mercury atoms bound to the sulfhydryl group as indicated by the fact that the line width was identical before and after dialysis.

It is possible that a sulfhydryl group could be situated at the active site and be masked by the zinc atom. This possibility was eliminated by performing a mercury titration on the apo enzyme as shown in Figure 1. The result is essentially the same as that for the native enzyme. This work therefore is completely consistent with the x-ray data of Lipscomb and his coworkers (3) who place the sulfur atoms at the outside of the molecule participating in a disulfide bridge. This location of the sulfur atoms precludes the possibility of sulfhydryl groups in the enzyme that are buried and inaccessible to mercuric ions.

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REFERENCES

1. J. E. Coleman and B. L. Vallee, J. Biol. Chem., 236, 2244 (1961).
2. K. A. Walsh, K. S. V. Sampath-Kumar, J. P. Bargetzi, and H. Neurath, Proc. Natl. Acad. Sci. U. S., 58, 2220 (1962).
3. W. N. Lipscomb, et al., Structure, Function, and Evolution in Proteins, Brookhaven Symposia in Biology: No. 21 (1968), pp. 24-90.
4. T. R. Stengle and J. D. Baldeschwieler, Proc. Natl. Acad. Sci. U. S., 55, 1020 (1966).
5. T. R. Stengle and J. D. Baldeschwieler, J. Am. Chem. Soc., 89, 3045 (1967).
6. B. L. Vallee, J. A. Rupley, T. L. Coombs, and H. Neurath, J. Biol. Chem., 235, 64 (1960).