Figure 12. The use of MITC in the sequenator will be the subject of a subsequent communication.

We have confirmed and extended Laursen's (1966) observation that MITC may be substituted for PITC in the Edman degradation. The major advantage which accrues is the ease of quantitative separation of the MTH derivatives of all the amino acids by gas–liquid partition chromatography. This gives increased confidence in the interpretation of the expected stepwise decrease of yield in the Edman degradation as well as making possible the analysis of mixed sequences.

References

Blomback, B., Blomback, M., Edman, P., and Hessel, B. (1966), *Biochim. Biophys. Acta 115*, 371.
Edman, P. (1950), *Acta Chem. Scand. 4*, 277, 283.
Edman, P. (1956), *Acta Chem. Scand. 10*, 761.
Edman, P., and Begg, G. (1967), *European J. Biochem. 1*, 80.
Edman, P., and Sjöquist, J. (1959), *Acta Chem. Scand. 10*, 1507.
Gray, W. R. (1967), *Methods Enzymol. 11*, 469.
Gray, W. R. (1968), *Nature 220*, 1300.
Harman, R. G., Patterson, J. L., and Vandenheuval, W. J. A. (1968), *Anal. Biochem. 25*, 452.
Konigsberg, W. (1967), *Methods Enzymol. 11*, 461.
Laursen, R. A. (1966), *J. Am. Chem. Soc. 88*, 5344.
Niall, H. D., and Potts, J. I., Jr. (1969), Proceeding of the 1st American Peptide Symposium, New York, N. Y., Marcel Dekker (in press).
Pisano, J. J., and Bronzert, T. J. (1969), *Fed. Proc. 28*, 661.
Pisano, J. J., Vandenheuval, W. J. A., and Horning, E. C. (1962), *Biochem. Biophys. Res. Commun. 7*, 82.
Richards, F. F., Barnes, W. T., Lovins, R. E., Salomone, R., and Waterfield, M. D. (1969), *Nature 221*, 1241.
Smith, G. P. (1969), Ph.D. Thesis, Harvard University, Cambridge, Mass.
Spackman, D. M., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stepanov, V. M., and Krivstov, V. F. (1965), *J. Gen. Chem. USSR 35*, 53, 556, 988.
Toi, K., Bynum, E., Norris, E., and Itano, H. A. (1965), *J. Biol. Chem. 240*, 3455.
Van Orden, H. O., and Carpenter, J. H. (1964), *Biochem. Biophys. Res. Commun. 14*, 339.

Perturbations of the Proton Magnetic Resonance Spectra of Conalbumin and Siderophilin as a Result of Binding Ga^{3+} or Fe^{3+}*

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ABSTRACT: Conalbumin binds specifically two Ga^{3+} per protein molecule with the ionization of four tyrosyl side chains, resulting in a shift to higher field of the aromatic region of the proton magnetic resonance spectrum. In contrast, binding of Fe^{3+} to conalbumin or siderophilin (transferrin) results in a decrease in proton magnetic resonance signal intensity in the aromatic region of the protein. This finding is consistent with the paramagnetic broadening or shift of the resonance of tyrosyl side chains specifically coordinated to high-spin Fe(III).

The hypothesis that phenolate side chains of certain tyrosyl residues serve as ligands in iron binding proteins such as conalbumin (Warner and Weber, 1953) from hen's egg white and siderophilin (transferrin) from human blood plasma (Hazen, 1962) has been substantiated by spectral (Wishnia *et al.*, 1961; Hazen, 1962; Tan and Woodworth, 1969a) and chemical modification studies (Line *et al.*, 1967; Komatsu and Feeney, 1967). As another probe of the effect of metal binding on these proteins, we have studied the interaction of conalbumin with Ga^{3+} and Fe^{3+} and of siderophilin with Fe^{3+} by proton magnetic resonance. The binding of Ga^{3+} results in shifts to high field of the resonances of aromatic protons in the bonding tyrosyl residues, whereas the binding of paramagnetic Fe^{3+} results in loss of signal intensity in the aromatic region owing to broadening or large shifts of the bonding tyrosyl signals.

Materials and Methods

Chemicals were reagent grade and were used without further purification. Deionized water was used to make all solutions and dilutions. Glassware was freed of iron by soaking in 1 mM EDTA—0.01 M sodium acetate (pH 4.5), then rinsing with deionized water. Standard iron solutions (0.020 M) were made by dissolving $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in

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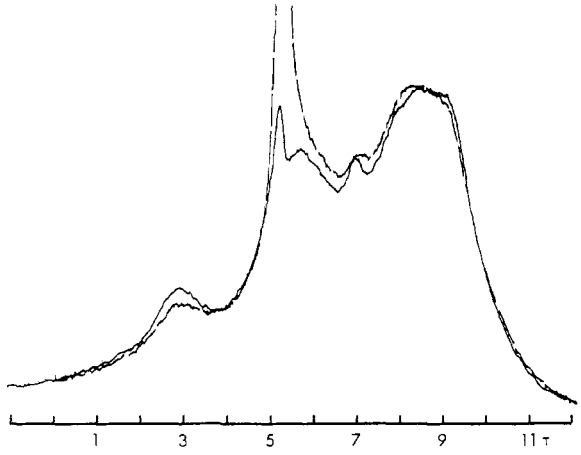

FIGURE 1: Proton magnetic resonance spectra at 100 MHz of 2.1 mM conalbumin (continuous line) and iron conalbumin (broken line) in 0.15 M KCl–0.005 M $NaHCO_3$, D_2O, pD 7.3, 30°.

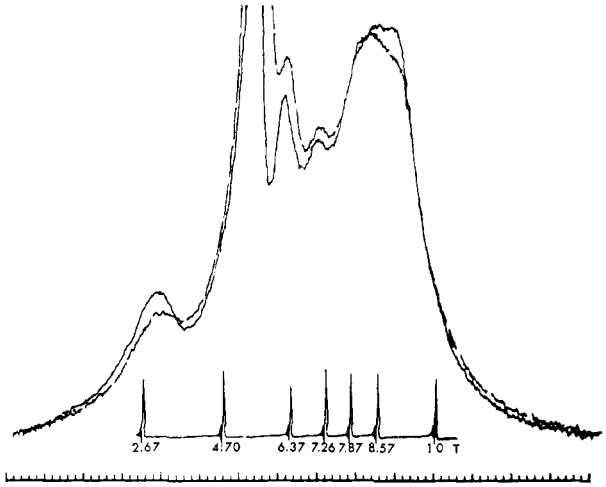

FIGURE 2: Proton magnetic resonance spectra at 60 MHz of 1.5 mM siderophilin (continuous line) and iron siderophilin (broken line) in 0.15 M KCl–0.01 M $KHCO_3$, D_2O, pD 8, 50°. External calibration standard contained tetramethylsilane, cyclohexane, acetone, trichloroethane, *p*-dioxane, CCl_2H_2, and $HCCl_3$ in CCl_4.

0.01 N HCl. Standard Ga^{3+} solutions (0.020 M) were made by dissolving gallium metal in a small volume of aqua regia, freeze drying the solution, and dissolving the residue in 0.01 N HCl.

Conalbumin was prepared as previously described (Woodworth and Schade, 1959) with the modification that the protein was chromatographed on CM-Sephadex C-50, rather than on CM-cellulose.

Siderophilin was isolated substantially as described (Roberts *et al.*, 1966) with the modification that all chromatography, save the last, was carried out with linear concentration gradients of ammonium bicarbonate (pH 8.0). The last chromatographic step employed the described phosphate–borate buffer in order to separate siderophilin from contaminating hemopexin. Iron was removed from purified iron–siderophilin by dialysis against a few changes of 1 mM nitrilotriacetate–0.2 M sodium phosphate (pH 6.2). The buffer was then removed by dialysis of the protein solution against several changes of deionized water.

The protein solutions were concentrated by lyophilization and dissolution of the residue in D_2O. The cycle was repeated once or twice in order to ensure equilibration of the protein with D_2O (99.7%) and maximal removal of H_2O. Final protein concentrations were usually *ca.* 2 mM.

Proton magnetic resonance spectra were recorded with 60-MHz (JEOL C-60HL), 100-MHz (JEOL JNM-4H-100), and 220-MHz (Varian HR-220) instruments, at temperatures ranging from 12 to 50°. In some studies protein samples were measured in a nuclear magnetic resonance tube with a Thunberg tube sealed onto the top. The sample could then be mixed with dried metal salts and potassium carbonate, contained in the top bulbs of the Thunberg tube, under vacuum or an O_2- and CO_2-free atmosphere.

Results

We have titrated conalbumin, in 0.10 M KCl–0.005 M $NaHCO_3$–CO_2 (pH 7.3), with Ga^{3+} and found difference spectral maxima at 243 and 294 nm, characteristic of metal ion binding by tyrosyl residues (Tan and Woodworth, 1969a). A plot of absorbance *vs.* Ga^{3+} added gave an end point at 3.5 μl of 0.020 M Ga^{3+} added to 2.0 ml of 0.018 mM conalbumin, *i.e.*, after specific binding of 2 moles of Ga^{3+}/mole of conalbumin. The $\Delta\epsilon_{243}$ of 4.1×10^4 for this reaction indicates that four tyrosyl residues are ionized per protein molecule on binding two Ga^{3+} (for tyrosyl phenol ionization in conalbumin $\Delta\epsilon_{240}$ is *ca.* 1×10^4; Tan and Woodworth, 1969a). The binding of Ga^{3+} to human siderophilin and inhibition of this binding by Fe^{3+} have been reported (Hartman and Hayes, 1967). Thus Ga^{3+} and Fe^{3+} bind to these proteins in a very similar way.

The proton magnetic resonance spectra of conalbumin and iron conalbumin at 100 MHz are shown in Figure 1, and the 60-MHz spectra of siderophilin and iron siderophilin in Figure 2. For both proteins iron binding results in (i) pronounced loss in signal intensity in the aromatic region, τ 2.0–3.5, particularly at about τ 2.9; (ii) a large increase in the residual water signal indicating that the water signal is no longer saturable; (iii) small reproducible changes in the τ 7 and 9 regions. All the changes are highly reproducible at both 60 and 100 MHz for both proteins. No significant changes were observed in the 100-MHz spectrum of conalbumin on the binding of gallium.

The spectra of the aromatic protons of conalbumin, iron conalbumin, and gallium conalbumin at 220 MHz are shown in Figure 3. There is little increase in the resolution of the protein absorption at this higher frequency probably because of the high molecular weight (McDonald and Phillips, 1969a) of this protein (76,000; Warner and Weber, 1953). However through the greater separation of the protein resonances from the residual HDO resonance it is now possible to see a clear shoulder on binding gallium, τ 3.8, while the binding of iron produces no comparable shoulder.

In a study of the ionization of phenol in D_2O and on addition of NaOD we observe a shift to high field of the *meta* protons, 0.2 ppm, and of 0.4 ppm for the *ortho* protons.

Aromatic region spectra (Figure 3) were simulated on the basis of data for random coil proteins (McDonald and Phillips, 1969b), using a residue half line width of 15 Hz

and the amino acid composition of conalbumin (Wenn and Williams, 1968). We assumed that Ga^{3+} shifts the resonances of four tyrosyl phenol side chains per protein molecule, whereas Fe^{3+} removes these resonances entirely from the aromatic region (Caughey *et al.*, 1968) along with the resonances of two tryptophanyl residues (Tan and Woodworth, 1969b). Coordination of histidyl side chains to the bound metal ions was neglected. Although we could expect only a rough approximation, the simulated spectra match the observed spectra well, especially the τ 3.8 shoulder in gallium–conalbumin and the reduction of intensity in the iron conalbumin spectrum which is best seen at 100 MHz.

Discussion

Our aim was to demonstrate the binding of tyrosyl side chains of conalbumin and siderophilin to Fe^{3+} by means of proton magnetic resonance spectra, for although difference spectra and chemical modification studies of iron binding by these proteins have implicated tyrosyl side chains as ligands, one might advance the argument that the changes observed were merely due to a conformational change which occurs when iron binds to the protein (Fuller and Briggs, 1956; Bezkorovainy, 1967; Tan and Woodworth, 1969b). Our observation that iron binding by the proteins results in a marked loss of signal intensity in the aromatic region is consistent with a large broadening or shift of phenolate resonances (Caughey *et al.*, 1968), and definitely implies coordination of Fe^{3+} to tyrosyl residues. In addition the experiments with Ga^{3+} demonstrate that these coordinating tyrosyl residues ionize when trivalent metal ions are specifically bound by the protein. Consistently observed changes in the τ 7 and 9 regions of the proton magnetic resonance spectra of conalbumin and siderophilin on iron binding reflect conformational changes elsewhere in the proteins.

The increased relaxation rate for solvent water caused by iron bound to these proteins has been reported (Aisen *et al.*, 1966). In contrast to those authors' findings of similar water relaxation rates for iron conalbumin with or without bound bicarbonate, we find that the iron bicarbonate–conalbumin complex relaxes the proton magnetic resonance signal of solvent water much faster than does iron conalbumin with no bound bicarbonate. A detailed study of this phenomenon, using spin-echo techniques, has already confirmed this observation, and will be reported elsewhere.

The approach we have taken here of contrasting the effects on the proton magnetic resonance spectra of proteins by the specific binding of a paramagnetic and a diamagnetic ion of similar chemical behavior should prove valuable in the analysis of the metal binding ligands of other proteins and metalloenzymes. Initial observations on carbonic anhydrase have been made in this laboratory (Oxford). While this work was in progress, a report of the use of Co^{2+} as a perturbing center in the proton magnetic resonance spectra of proteins appeared (McDonald and Phillips, 1969c).

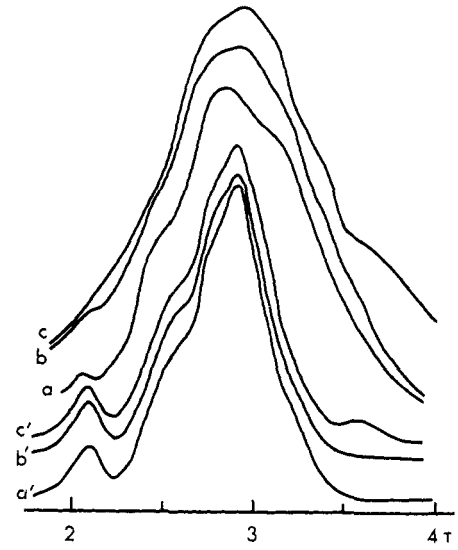

FIGURE 3: Proton magnetic resonance spectra at 220 MHz of the aromatic region of 2.1 mM conalbumin (a), gallium conalbumin (b), and iron conalbumin (c), in 0.15 M KCl–0.01 M $NaHCO_3$–CO_2, D_2O, pD 7.3, 12°. Signal to noise ratios were improved by accumulation of 16–25 scans by a computer of average transients, so amplitudes cannot be quantitatively compared. The respective simulated spectra (a', b', and c') are included for comparison. Note that the baselines have been shifted for the sake of definition. If the base lines of c' and a' are brought into coincidence, the area under c' is seen to be appreciably less than that under a'.

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References

Aisen, P., Leibman, A., and Reich, H. A. (1966), *J. Biol. Chem. 241*, 1666.

Bezkorovainy, A. (1967), *Biochim. Biophys. Acta 127*, 535.

Caughey, W. S., Wüthrich, K., and Shulman, R. G. (1968), 3rd International Conference on Magnetic Resonance in Biological Systems, Warrenton, Va., Oct 1968.

Fuller, R. A., and Briggs, D. R. (1956), *J. Am. Chem. Soc. 78*, 5253.

Hartman, R. E., and Hayes, R. L. (1967), *Fed. Proc. 26*, 780.

Hazen, E. E., Jr. (1962), Ph.D. Thesis, Harvard University, Cambridge, Mass.

Komatsu, S. K., and Feeney, R. E. (1967), *Biochemistry 6*, 1136.

Line, W. F., Grohlich, D., and Bezkorovainy, A. (1967), *Biochemistry 6*, 3393.

McDonald, C. C., and Phillips, W. D. (1969a), *Biochem. Biophys. Res. Commun. 35*, 492.

McDonald, C. C., and Phillips, W. D. (1969b), *J. Am. Chem. Soc. 91*, 1513.

McDonald, C. C., and Phillips, W. D. (1969c), *Biochem. Biohys. Res. Commun. 35*, 43.

Roberts, R. C., Makey, D. G., and Seal, U. S. (1966), *J. Biol. Chem. 241*, 4907.

Tan, A. T., and Woodworth, R. C. (1969a), *Biochemistry 8*, 3711.

Tan, A. T., and Woodworth, R. C. (1969b), *J. Polymer*

Sci., Part C (in press). Preprints of papers, IUPAC International Symposium of Macromolecular Chemistry, Toronto, Sept 1968.
Warner, R. C., and Weber, I. (1953), *J. Am. Chem. Soc. 75*, 5094.
Wenn, R. V., and Williams, J. (1968), *Biochem. J. 108*, 69.
Wishnia, A., Weber, I., and Warner, R. C. (1961), *J. Am. Chem. Soc. 83*, 2071.
Woodworth, R. C., and Schade, A. L. (1959), *Arch. Biochem. Biophys. 82*, 78.

1-Amino-1,3-dicarboxycyclohexane (Cycloglutamic Acid). A New Glutamic Acid Analog and a Substrate of Glutamine Synthetase*

Jerald D. Gass and Alton Meister

ABSTRACT: The ability of ovine brain glutamine synthetase to utilize α-methyl-L-glutamate, *threo*-β-methyl-D-glutamate, and *threo*-γ-methyl-L-glutamate (but not other monomethyl-substituted glutamates) suggested that 1-amino-1,3-dicarboxycyclohexane (in which a chain of three methylene groups is introduced between the α- and γ-carbon atoms of glutamate) might be a substrate of the enzyme. 1-Amino-1,3-dicarboxycyclohexane (designated here by the trivial term cycloglutamic acid) was synthesized by hydrolysis of 3-carboxycyclohexane-1-spiro-5′-hydantoin (prepared by reacting 3-carboxycyclohexanone with ammonium carbonate and sodium cyanide).

The *cis* and *trans* racemic forms of cycloglutamic acid were obtained separately and tested for activity with the enzyme. Close to 50% of the racemic *cis* form was utilized by the enzyme in amide and hydroxamate synthesis, while the *trans* form was not active. The enzymatically synthesized amide was isolated and compared with the chemically synthesized product. The K_m and V_{max} values for *cis*-cycloglutamate in hydroxamate synthesis were similar to the corresponding values for glutamate. *cis*-Cycloglutamate (but not the *trans* isomer) can assume a diequatorial conformation equivalent to the extended conformation of glutamate. The fact that *cis*-cycloglutamate, whose 5-carbon chain is much more restricted in movement than that of glutamate, is a good substrate of glutamine synthetase affords strong support for the hypothesis that L-glutamate binds to the active site of the enzyme in the extended conformation.

The unusual substrate specificity of ovine brain glutamine synthetase is such that of the ten possible glutamate derivatives in which a methyl group is introduced into the glutamate carbon chain, only three are substrates: α-methyl-L-glutamate, *threo*-β-methyl-D-glutamate, and *threo*-γ-methyl-L-glutamate (Kagan *et al.*, 1965; Kagan and Meister, 1966a,b; Meister, 1968) (Figure 1). These findings and the observations that both the L and D isomers of glutamate and α-aminoadipate are substrates (Wellner *et al.*, 1966), and that β-glutamate is enzymatically converted into D-β-glutamine (Khedouri and Meister, 1965), have led to an hypothesis concerning the conformation of these substrates on the enzyme. According to this hypothesis, (a) the carbon chain of L-glutamate is oriented on the enzyme in the fully (or almost fully) extended conformation in which the α-hydrogen atom of the substrate is directed away from the enzyme, (b) D-glutamate attaches to the enzyme in an extended conformation in which the α-hydrogen atom of this substrate is directed toward the enzyme.

The finding that the γ-*threo* and α-hydrogen atoms of L-glutamate can be replaced by methyl groups with retention of enzymatic susceptibility suggested the attractive possibility of constructing a cyclohexane ring by introducing a chain of three methylene groups connecting carbon atoms 2 and 4 of the glutamate carbon chain (Figure 2). The *cis*-L isomer of the resulting cyclohexane amino acid can exist in a form possessing a relatively rigid five-carbon chain identical with that of the extended conformation of L-glutamic acid; the position of the cyclohexane chain is entirely in the region in which methyl group substitutions that are consistent with retention of enzymatic susceptibility can be made. In this communication the synthesis of 1-amino-1,3-dicarboxycyclohexane[1] is described. Close to 50% of the racemic *cis* isomer is utilized by glutamine synthetase. The findings offer strong support for the hypothesis that L-glutamate attaches in the extended conformation to the active site of the enzyme.

Experimental Section

Methods and Materials. *m*-Methoxybenzoic acid was purchased from Distillation Products, Inc. Glutamine synthetase was prepared according to the method of Ronzio

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[1] This will be designated by the trivial term cycloglutamic acid.