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Studies on the Conformational Isomers of Deoxycytidylate Aminohydrolase*

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ABSTRACT: Binding of regulatory ligands to deoxycytidylate aminohydrolase induces in the enzyme conformational changes that have been investigated by means of titration of thiol groups, reactivity of amino acid residues, difference spectra, quenching of fluorescence, and time rate of inactivation with proteolytic enzymes.

The data reported here indicate the existence of at least three conformational isomers of deoxycytidylate aminohydrolase, namely, the conformation of the enzyme with no ligands, the conformation of the enzyme–dCTP–Mg complex, and the conformation of the enzyme–dTTP–Mg complex.

Deoxycytidylate aminohydrolase is a typical allosteric enzyme. Its activity is regulated by highly specific allosteric effectors (Geraci *et al.*, 1967; Scarano *et al.*, 1967a). Deoxycytidine triphosphate is the allosteric activator and deoxythymidine triphosphate is the allosteric inhibitor. In the presence of Mg ions the enzyme binds four molecules of dCTP or four molecules of dTTP at saturating concentrations of the activator or of the inhibitor, respectively. With a homogeneous preparation of deoxycytidylate aminohydrolase we have described homotropic and heterotropic effects for the substrate and for the allosteric and isosteric effectors (Rossi *et al.*, 1967; Scarano *et al.*, 1967b), and have shown that no change in molecular weight of the enzyme occurs on binding of the regulatory ligands (Geraci *et al.*, 1967; Scarano *et al.*, 1967a). The enzyme with no ligands, enzyme–dCTP–Mg complex, and enzyme–dTTP–Mg complex all have a molecular weight of 1.2×10^5. Thus, monomer–polymer equilibrium plays no role in the regulation of the activity of deoxycytidylate aminohydrolase. In previous papers we have reported kinetic experiments which suggest the occurrence

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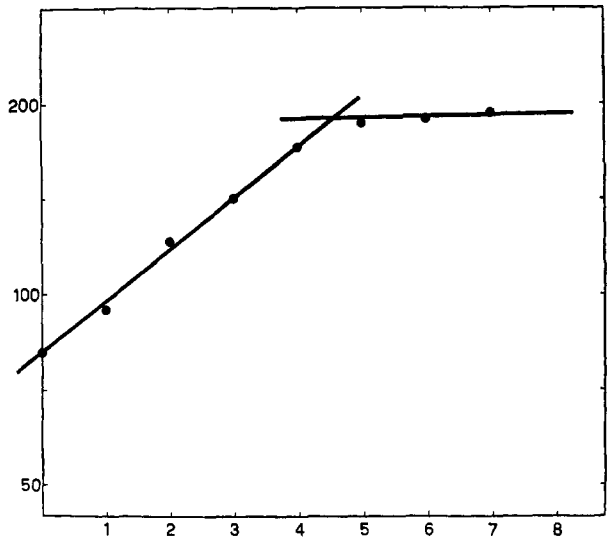

FIGURE 1: Sulfhydryl groups determination by the Boyer method. Enzyme, 0.14 nmole in 0.2 ml; *p*-chloromercuribenzoate, 0.72 nmole/μl in 0.02 N NaOH. Abscissa, microliters of reagent added; ordinate, absorbancy at 255 nm.

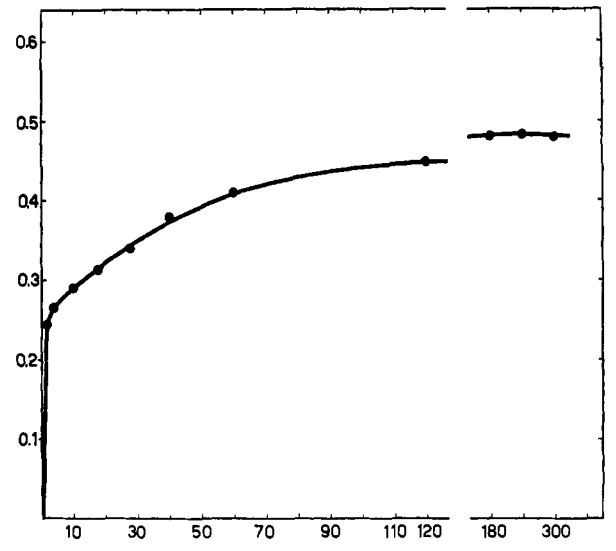

FIGURE 2: Reactivity of SH groups of dCMP-aminohydrolase in 4 M urea. The enzyme at a concentration of 174 μg/ml equivalent to 1.45 nmoles was incubated at 22° for 30 min in 4 M urea, 0.03 M phosphate buffer (pH 8). Abscissa, time in minutes after the addition of the Ellman reagent; ordinate, absorbancy at 412 nm. T = 22°.

of at least three conformational isomers of the enzyme (Rossi and Scarano, 1969; Rossi *et al.*, 1970).

Monod, Wyman, and Changeux have proposed a model to explain the regulation of the activity of allosteric enzymes based on the occurrence of at least two conformational isomers (Monod *et al.*, 1965).

To investigate the number of the conformational isomers underlying the regulation of the activity of deoxycytidylate aminohydrolase different experimental approaches have been pursued and will be reported here. The results obtained strongly suggest that at least three conformational isomers of dCMP-aminohydrolase underlie the regulation of its activity.

Materials and Methods

Deoxycytidylate aminohydrolase was prepared by a modification of the procedure described by Geraci *et al.* (1967) (M. Rossi *et al.*, in preparation). The enzyme activity was measured by the spectrophotometric assay previously described (Scarano *et al.*, 1967a). When the inactivation of the enzyme was studied as a function of time 5- or 10-μl aliquots were removed from the incubation mixture described in the legend and the enzyme activity was assayed. Proteolytic enzymes were obtained from Worthington. Titration of SH groups was made by the method of Boyer (1954) and by the method of Ellman (1959). *p*-Chloromercuribenzoic acid was obtained by Sigma Co. and was recrystallized twice from water. Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoate), was obtained from Aldrich Chem. Co. This reagent produces at pH 8 1 mole of 2-nitro-5-thiobenzoic acid/mole of thiol group. The colored anion has a molar extinction (ϵ × 13,600 M^{-1} × cm^{-1}) at 412 nm. Difference spectra were obtained with a PMQII Zeiss spectrophotometer equipped with a special cuvet holder, which has a reference compartment and a sample compartment. A front cuvet and a rear cuvet each of 10-mm light path are optically aligned in each compartment. Fluorescence measurements were performed with a Turner Model 210 spectrofluorometer at 22° using excitation and emission bandwidth of 100 Å.

Results

Thiol Groups Titration. On the basis of a molecular weight of 1.2 × 10^5 (Geraci *et al.*, 1967), 24 SH groups/mole of deoxycytidylate aminohydrolase are determined by the Boyer (1954) method (Figure 1). The same number of SH groups is measured in the presence of saturating concentrations of dCTP and of dTTP.

By using the Ellman reagent 24 SH groups/mole of enzyme are also determined when the enzyme is incubated for 30 min in 4 M urea before addition of the reagent. The reaction

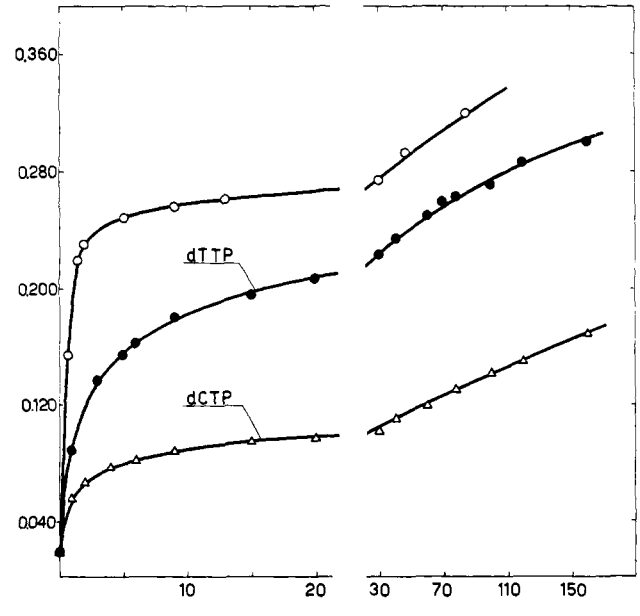

FIGURE 3: Effect of dCTP and dTTP on the reactivity of SH groups of dCMP-aminohydrolase. Enzyme concentration 184 μg/ml, equivalent to 1.53 nmole/ml, $MgCl_2$ 1 mM, and phosphate buffer 0.03 M (pH 8). Abscissa, time in minutes after the addition of the Ellman reagent; ordinate, absorbancy at 412 nm. (○) No addition; (●) 10 μM dTTP; (△) 10 μM dCTP. T = 22°

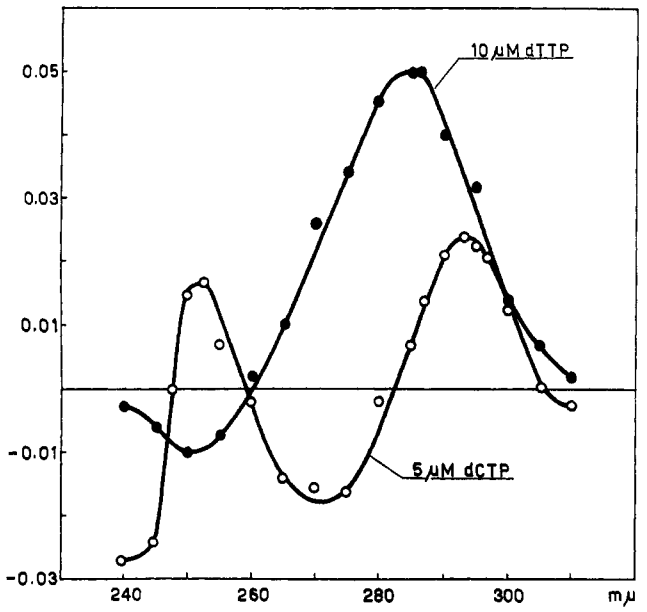

FIGURE 4: Differential spectra of dCMP-aminohydrolase in the presence of dCTP (○) and dTTP (●). The following procedure was used for measuring both spectra. The rear cuvets in each compartment of the special holder were filled with two 1-ml portions of a solution containing 460 μg/ml of the enzyme in 0.05 M Tris-HCl buffer, 1 mM $MgCl_2$ (pH 7.5). The two front cuvets were filled each with 1 ml of either 10 μM dCTP or 20 μM dTTP in the same buffer. Readings were taken at the wavelengths indicated by the points of the figure and they were in all instances inferior to $A = \pm 0.004$. The contents of the front and the rear cuvets of the sample compartment were accurately mixed and the absorbancy was measured again. These values were corrected of the values obtained before mixing. $T = 22°$.

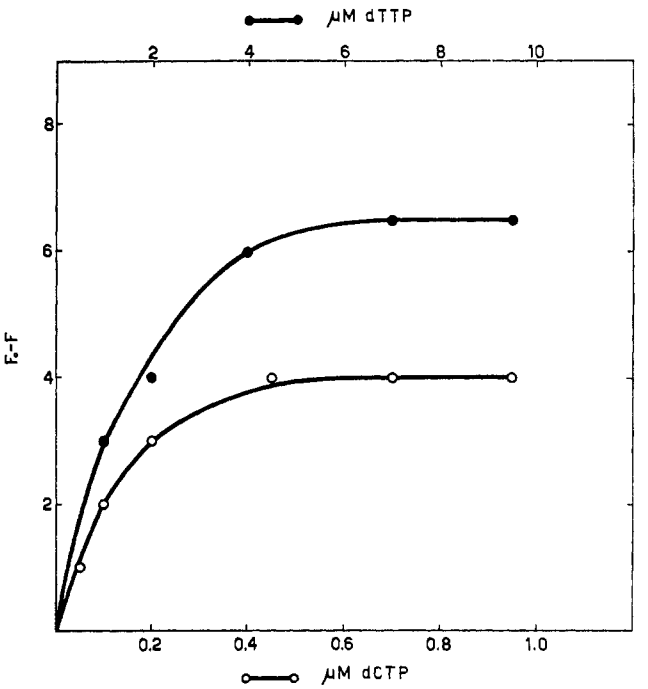

FIGURE 5: Quenching of fluorescence of dCMP-aminohydrolase by dCTP (○) and by dTTP (●). Excitation wavelength 280 nm and emission wavelength 340 nm. The incubation mixture contained 0.05 M Tris buffer, 0.5 mM $MgCl_2$ (pH 7.5). Enzyme concentration 25 μg/ml. $T = 22°$

is complete in 3 hr and an absorbancy value at 412 nm of 0.480 is obtained (Figure 2). However, without preincubation of the enzyme in 4 M urea, two types of SH groups, namely, the fast- and the slow-reacting SH groups, can be observed with the Ellman ragent (Figure 3). From the data shown it is possible by extrapolation at zero time to read the following absorbancy values at 412 nm; 0.240 for the free enzyme; 0.170 for the enzyme–dTTP–Mg complex; 0.080 for the enzyme–dCTP–Mg complex. These absorbancy values correspond to 12 fast-reacting SH groups/mole of the free enzyme, 8/mole of the enzyme–dTTP–Mg complex, and 4/mole of the enzyme–dCTP–Mg complex.

The existence in the absence of ligands of two conformations of the enzyme in equilibrium seems to be excluded by the fact that the enzyme with no ligands has the highest number of titrable SH groups. However, the data can be easily explained by the occurrence of three conformational isomers of deoxycytidylate aminohydrolase. In addition, the data indicate that in the absence of ligands almost 100% of the enzyme molecules is in one specific conformation. Another possible interpretation of the data is shielding of different number of SH groups of the enzyme molecule by the activatory ligands and by the inhibitory ligands. Under this assumption the time rate of titration of SH groups would have no relation with the conformation of the enzyme.

Ultraviolet Difference Spectra. The ultraviolet spectra of the enzyme–dTTP–Mg complex and of the enzyme–dCTP–Mg complex against the enzyme are shown in Figure 4. The enzyme–dCTP–Mg complex gives a difference spectrum different from that of the enzyme–dTTP–Mg complex. These spectra reflecting the nature of the environment surrounding tryptophan and tyrosine residue of the enzyme, indicate that dCTP and dTTP induce different conformations of the enzyme with respect to the conformation of the enzyme with no ligand. Moreover, the data cannot be explained on the assumption that in the reference cuvet there is a mixture of the activated and of the inhibited conformation because spectral regions occur in which both complexes have different but positive ΔA. The absorbancy at 280 nm of the enzyme–dTTP–Mg complex is higher than that of the enzyme with no ligand. At the same wavelength the enzyme–dCTP–Mg complex has the same absorbancy than the enzyme with no ligand. But between 280 and 305 nm both the enzyme–dTTP–Mg complex and the enzyme–dCTP–Mg complex have a higher absorbancy than the enzyme with no ligand. Thus the existence of three conformational isomers of the enzyme are required to explain the ultraviolet difference spectra.

Quenching of Fluorescence. Deoxycytidylate aminohydrolase shows excitation maximum at 280 nm and emission maximum at 340 nm in 0.05 M Tris-HCl buffer–0.5 mM $MgCl_2$ (pH 7.5), $T = 22°$. The fluorescence at 340 nm was measured after successive additions of the allosteric effectors. Both allosteric effectors quench the fluorescence and dCTP produces a different quenching from that of dTTP (Figure 5). This indicates at least three conformational isomers of the enzyme. The conformation of the enzyme in the absence of ligand gives a fluorescence higher than that of the enzyme–dCTP–Mg complex, and the conformation of the enzyme–dCTP–Mg complex a fluorescence higher than that of the enzyme–dTTP–Mg complex. The concentrations of the two allosteric effectors which produce half-maximum quenching of fluorescence are equal to those found by measuring the kinetics of the activation and the inhibition of the enzyme (Scarano *et al.*, 1967a). Further work is necessary to demonstrate whether the quenching of

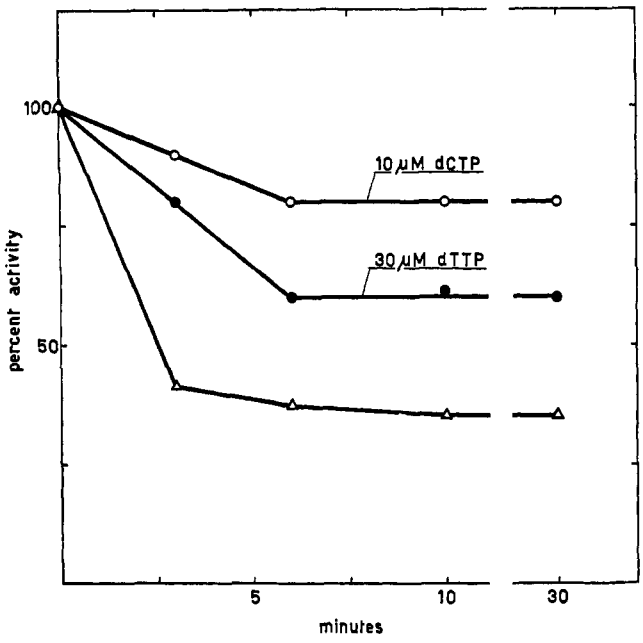

FIGURE 6: Time dependence of the action of *N*-bromosuccinimide on the activity of dCMP-aminohydrolase. The incubation mixture contained 0.1 M phosphate buffer, 1 mM $MgCl_2$ (pH 7.0). Enzyme concentration 51 μg/ml, *N*-bromosuccinimide 6.5 μM, and nucleotides as specified. $T = 22°$.

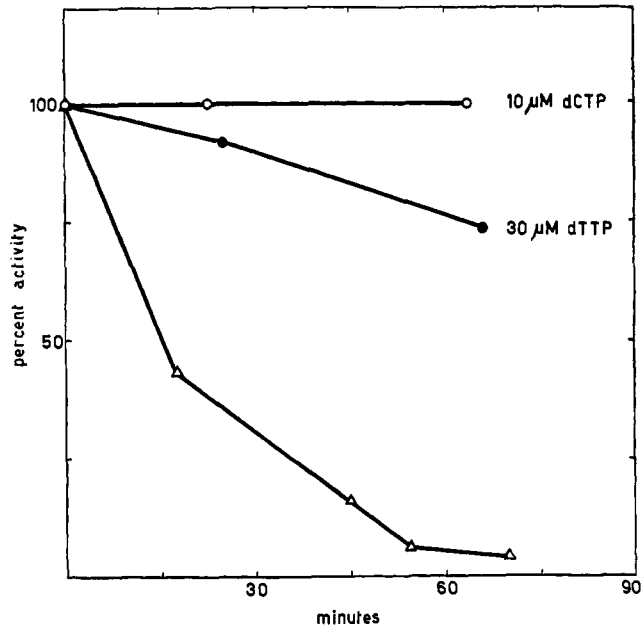

FIGURE 7: Time dependence of the action of carboxypeptidase B on the activity of dCMP-aminohydrolase. The incubation mixture contained 0.05 M Tris buffer (pH 7.5), 10 mM $MgCl_2$, dCMP-aminohydrolase concentration 125 μg/ml, carboxypeptidase B 350 μg/ml, and nucleotides as specified. $T = 38°$.

the fluorescence by the allosteric effectors follows a sigmoid kinetics as does the activation and the inhibition of the enzyme.

Inactivation by N-Bromosuccinimide. Figure 6 depicts the inactivation by *N*-bromosuccinimide of the enzyme, of the enzyme–dCTP–Mg complex, and of the enzyme–dTTP–Mg complex as a function of time. Under the conditions used *N*-bromosuccinimide oxidizes mainly the tryptophan residues. The enzyme in the absence of ligands has the highest inactivation rate and the enzyme–dCTP–Mg complex the lowest one.

Inactivation by Proteolytic Enzymes. By digesting deoxycytidylate aminohydrolase with the two exopeptidases, carboxypeptidases B and A, three different time rates of inactivation for the enzyme and for the complexes with effectors are found. This is shown in Figure 7 for carboxypeptidase B.

An identical result is obtained with three endopeptidases: chymotrypsin, trypsin, and nagarase. In Figures 8 and 9 the data obtained with chymotrypsin and trypsin are depicted. In all instances in which the digestibility of the enzyme by proteolytic enzymes was investigated the enzyme–dCTP–Mg complex was stable, the enzyme with no ligands had a very fast rate of inactivation, and the enzyme–dTTP–Mg complex an intermediate one.

Discussion

On the basis of kinetic experiments and binding studies we have previously suggested (Scarano *et al.*, 1968) that deoxycytidylate aminohydrolase is composed of four protomers. The enzyme molecule of mol wt 120,000 can bind at the same time four molecules of an isosteric ligand and four molecules of an allosteric ligand (Scarano *et al.*, 1967b). We have also proposed that changes of the conformation of the enzyme molecule underlie the regulation of the activity of dCMP-aminohydrolase (Rossi and Scarano, 1969). Monod *et al.* (1965) have assumed that the protomers of allosteric enzymes exist in at least two configurations and in addition they advanced the hypothesis of conservation of symmetry of the protein molecule undergoing allosteric transitions. The data presented in this paper cannot be explained on the basis of only two conformations but imply the existence of at least three conformational isomers of dCMP-aminohydrolase.

The changes of conformations caused by the regulatory

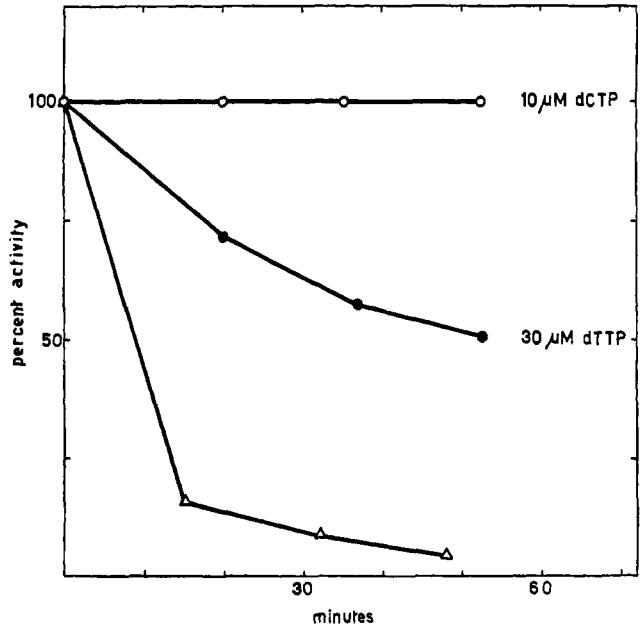

FIGURE 8: Time dependence of the action of chymotrypsin on the activity of dCMP-aminohydrolase. The incubation mixture contained 50 mM phosphate buffer (pH 7.4), 10 mM $MgCl_2$, 125 μg/ml of dCMP-aminohydrolase, 0.075 μg/ml of chymotrypsin, and nucleotides as specified. $T = 24°$.

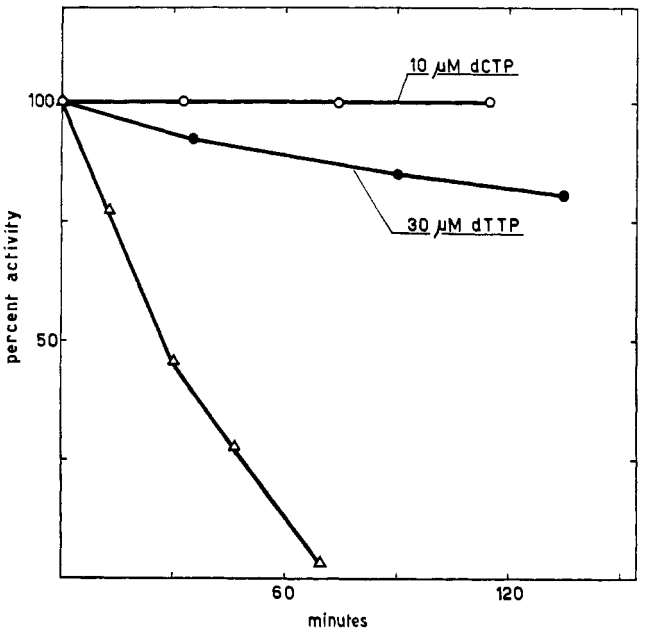

FIGURE 9: Time dependence of the action of trypsin on the activity of dCMP-aminohydrolase. The incubation mixture contained 50 mM phosphate buffer (pH 7.4), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 125 μg/ml of dCMP-aminohydrolase, 10 μg/ml of trypsin, and nucleotides as specified. $T = 32°$.

ligands are considerable. These changes involve: (1) cysteine residues as demonstrated by the time rate of titration of SH groups; (2) aromatic amino acids as shown by the ultraviolet difference spectra, by the quenching of fluorescence, and by the time rates of inactivation by *N*-bromosuccinimide and chymotrypsin; (3) the carboxyl-terminal amino acid or amino acids as suggested by the time rates of inactivation by carboxypeptidase; (4) arginines and lysines as indicated by the rate of inactivation by trypsin.

All these considerable changes in so many different amino acid residues cannot be caused by a shielding phenomenon, but can be explained by the existence of three conformational isomers of dCMP-aminohydrolase.

Kirtley and Koshland (1968), on the basis of a theoretical analysis of the data by Scarano *et al.* (1962, 1967a) and by Geraci *et al.* (1967), have also proposed that there are at least three conformational isomers of dCMP-aminohydrolase. However, a different interpretation has been discussed by Whitehead (1970).

Whether symmetry is conserved or not in the changes of conformations of dCMP-aminohydrolase cannot be decided on the basis of the present data. However, extension of the experiments reported here will permit testing of the validity of the symmetry mechanism. For instance, determinations of ultraviolet spectral changes and of the quenching of fluorescence as a function of the concentrations of the regulatory ligands will certainly give pertinent information.

Acknowledgments

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