13. P. Langen and G. Etzold, *Biochem. Z.* **339**, 190 (1963).
14. M. Zimmerman and J. Seidenberg, *J. Biol. Chem.* **239**, 2618 (1964).
15. W. H. Prusoff, *Biochim. Biophys. Acta* **32**, 295 (1959).
16. G. Etzold and P. Langen, *Chem. Ber.* **98**, 1988 (1965).
17. Z. Dische, *Mikrochemie* **8**, 4 (1930).
18. W. H. Prusoff, *Biochim. Biophys. Acta* **39**, 327 (1960).
19. W. H. Prusoff, W. L. Holmes and A. D. Welch, *Cancer Res.* **13**, 221 (1953).

Alanine Racemase from *Staphylococcus aureus*: Conformation of Its Substrates and Its Inhibitor, D-Cycloserine

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SUMMARY

D-Cycloserine is a competitive inhibitor of alanine racemase for which its K_i is 100 times smaller than the K_m for either of the substrates, D- and L-alanine. L-Cycloserine, however, does not inhibit this enzyme. A hypothesis is proposed, based on molecular models, that D-cycloserine has the conformation required of the substrates on the enzyme surface. L-Cycloserine cannot have this conformation.

Alanine racemase (1) is the enzyme which interconverts L-alanine and D-alanine in bacteria. This enzyme in *Staphylococcus aureus* is competitively inhibited by D-cycloserine and is one of the targets for the antibacterial action of this antibiotic (2). Further study of this inhibition has led to a hypothesis regarding the conformation of the two substrates on the enzyme surface.

For these studies alanine racemase was purified 100-fold from the supernatant solution prepared after sonic disintegration of *S. aureus*. The pH optimum of the enzyme was 8–9. The K_m for either L-alanine or D-alanine was 4–6 mM when measured in phosphate buffer. The activity of the preparation was not enhanced by the addition of pyridoxal phosphate. By contrast, the enzyme obtained from *S. fecalis* (1) was readily resolved from its cofactor by purification. The *S. aureus* racemase was, however, inhibited by a number of reagents which react with carbonyl groups (Table 1), suggesting the probable presence of pyridoxal phosphate in the enzyme.

TABLE 1

Inhibitor	K_i (mM)	Type of inhibition
NH_2OH	0.012	Competitive
NH_2NH_2	0.10	Competitive
KCN	>10	No inhibition
Semicarbazide	>10	No inhibition
β-Aminoxy-D-alanine	0.04	Competitive
D-Cycloserine	0.05	Competitive
L-Cycloserine	≫10	No inhibition

A surprising result of this study was the finding that, although the K_i for D-cycloserine was about 0.05 mM whether the reac-

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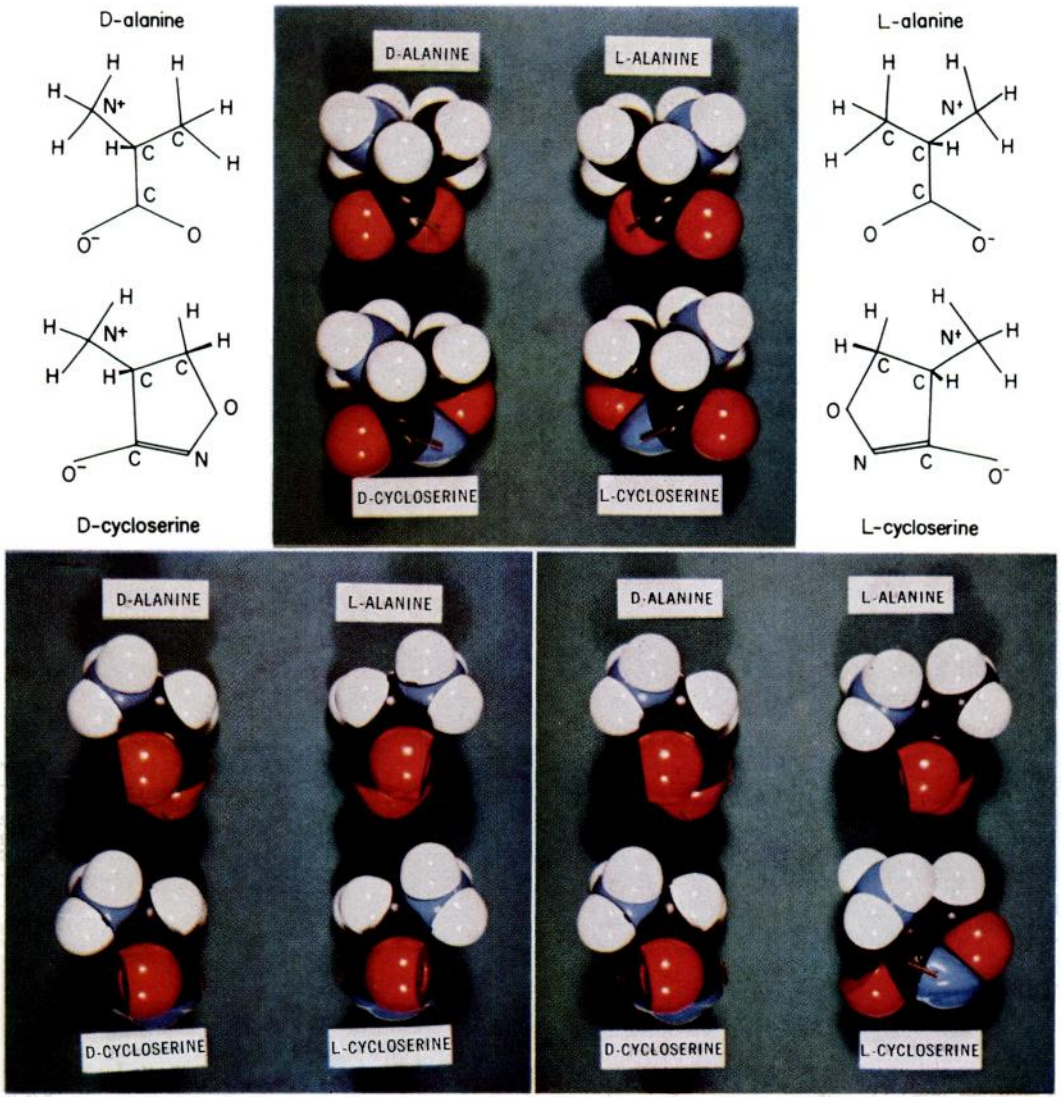

FIG. 1. *Molecular models (Stuart and Briegleb) of* D- *and* L-*alanine and* D- *and* L-*cycloserine*

The models were built in the zwitterion forms.

At top, the models are photographed looking down on the hydrogen atom on the asymmetric carbon atoms. Note the closed ring in the cycloserines. The line drawings were made from projections of photographs of the models.

In bottom left, the models have been turned 90°. The asymmetric carbon atom is at the center, and three of the groups which substitute it can be seen. Note that the dihedral angle involving the positive amino group and the negative oxygen is the same in D-alanine and D-cycloserine, but opposite in sign in L-alanine and L-cycloserine.

In bottom right, L-alanine has been rotated so that the dihedral angle is identical to that in D-alanine. The methyl group of L-alanine is now in the same position as the hydrogen atom on the asymmetric carbon atom of D-alanine. In the L-cycloserine model, with the amino group in the same position as in L-alanine, the negative oxygen is at the back of the model and cannot be rotated into the position in which it is found in the other 3 molecules.

In these illustrations carbon atoms are black, oxygen atoms are red, nitrogen atoms are blue, and hydrogen atoms are white.

tion was measured in the direction D-alanine → L-alanine or L-alanine → D-alanine (2), no significant inhibition was observed at 10 mM L-cycloserine, the highest concentration tested. On the other hand, the K_m values for D-alanine and L-alanine were virtually identical (about 5 mM). Several different samples of D- and L-cycloserine were employed in these studies, and the authenticity of several of the L-cycloserine samples was demonstrated by their action as competitive inhibitors of L-alanine-α-ketoglutarate (L-glutamate-pyruvate) transaminase from pig heart (3) ($K_i = 2 \times 10^{-6}$ M). Moreover, in studies of the nonenzymic reaction of D- or L-cycloserine with pyridoxal phosphate, both behaved identically (4, 5).

The apparent anomaly can be explained by considering the fact that D-alanine and L-alanine must have the same conformation on the enzyme surface, assuming that there is only a single active site in the enzyme. This conformation is presumably that present in D-cycloserine, since this compound is bound more tightly to the enzyme than is either of the natural substrates. It is possible for D-alanine and L-alanine to have the same conformation with respect to the functional groups, i.e., the amino and carbonyl group, as is found in D-cycloserine (Fig. 1). L-Cycloserine, however, cannot be rotated into this conformation, because the closure of the ring in the antibiotic prevents rotation at the carbon-carbon bonds.

The failure of L-cycloserine to inhibit alanine racemase, in contrast to D-cycloserine, points to a plausible hypothesis regarding the conformation required for both of the substrates, L-alanine and D-alanine, on the enzyme surface. It also provides an explanation of the fact that the K_i for the inhibitor (D-cycloserine) is 100 times smaller than the K_m for D- or L-alanine. These constants indicate that the antibiotic is bound to the enzyme 100 times more effectively than the natural substrates. The antibiotic is fixed in the conformation preferred by the enzyme, in contrast to the substrates, which have many possible conformations. Consequently, the concentration of D-cycloserine in the conformation required by the enzyme is much greater than the concentration of either of the substrates in the corresponding conformation. Moreover, once it is in the substrate binding site, the antibiotic cannot rotate and is, therefore, relatively rigidly fixed there. The possibility of rotation of the substrate presumably permits it to come off the enzyme surface more readily. Thus, the antibiotic both could more readily enter the substrate binding site and, once in it, would come out of it with more difficulty.

The conformational situation described here is akin to the conformational problem with regard to L- and D-glutamic acid or α-methyl-L- and α-methyl-D-glutamic acid as substrates for glutamine synthetase, recently discussed by Kagan *et al.* (6). In the absence of the bulky methyl group, L- and D-glutamic acid can assume a similar conformation with respect to the functional groups, and hence both are substrates for the enzyme. Similarly, D- and L-alanine as substrates for alanine racemase must have the same conformation with respect to the functional groups. Further studies of the interaction of the substrates and of D-cycloserine with alanine racemase are in progress.

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REFERENCES

1. W. A. Wood and I. C. Gunsalus, *J. Biol. Chem.* **190,** 403 (1951).
2. J. L. Strominger, E. Ito, and R. H. Threnn, *J. Am. Chem. Soc.* **82,** 998 (1960).
3. R. M. Azarkh, A. E. Braunshtein, T. S. Paskhina, and H. Ting-Sen, *Biokhimiya* **25,** 954 (1960).
4. U. Roze and J. L. Strominger, *Federation Proc.* **22,** 423 (1963).
5. U. Roze, Ph.D. Thesis, submitted to the Graduate School of Washington University, St. Louis, Missouri, 1964.
6. H. M. Kagan, L. R. Manning and A. Meister, *Biochemistry* **4,** 1063 (1965).