Effect of solvent on exchange rate of valinomycin with its KCNS complex

The lactate methyl peaks were recorded eight times with sweep width of 0.9 ppm. The width at half maximum height ($\Delta v$) was taken of the high field peak of the lactate methyl proton doublets of valinomycin or the downfield peak of the lactate methyl doublet of valinomycin-KCNS. The other components of these doublets are superimposed when the sample is partially titrated. Radio frequency power was kept below the saturation level. The exchange rates for the species observed (valinomycin or valinomycin-KCNS) were calculated from Equation 2. Apparent bimolecular rate constants were calculated from the relation

$$-\frac{d[\text{valinomycin}]}{dt} = k_{app}[\text{valinomycin}][\text{valinomycin-KCNS}]$$

The total valinomycin concentration was 25 mg per ml (≈ 22.7 mM).

Table I shows that in CH$_3$OH-CDC$_3$, exchange rates, i.e. $1/\tau_{\text{valinomycin}}$, of about 2 sec$^{-1}$ occur while in pure CDC$_3$ the lack of detectable lactate resonance broadening fits an upper limit of turnover of 0.2 sec$^{-1}$. This conforms well with the mobile carrier hypothesis over the ion channel hypothesis, for the mechanism of valinomycin-mediated ion transport in particular and ionophore-mediated ion transport in general.

**REFERENCES**


**Carboxyl Group Modification and the Activity of Lysozyme**

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**SUMMARY**

Modification of the carboxyl groups in hen's egg white lysozyme by the carbodiimide-nucleophile procedure developed in this laboratory has shown that all of the carboxyl groups except glutamic 35 can be modified under normal assay conditions. The presence of the substrate protects the aspartic acid residue 52 so that an enzyme with over 50% activity is obtained in which all carboxyls except aspartic 52 and glutamic 35 have been converted to the

$$\text{NH}_{2}\text{CH}_{2}\text{SO}_{3}^-$$

derivatives. Subsequent treatment after the removal of substrate leads to concomitant modification of aspartic 52 and loss of enzyme activity. These results eliminate all carboxyl residues except glutamic 35 and aspartic 52 as potential catalytic groups or essential binding groups. They add strong support to the hypothesis of Phillips and his coworkers, deduced from crystallographic studies, that these residues are involved in the catalytic action.

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other carboxyl groups appear to be on the surface of the protein molecule in terms of accessibility to reagent but individual residues vary in reactivity both in the carboxyl activation step and in the subsequent nucleophilic displacement on the activated carboxyl.

The structure of hen's egg white lysozyme has been elucidated by the elegant crystallographic studies of Phillips et al. (1-3). They found that the 11 free carboxyl groups (2 glutamic acid residues, 8 aspartic acid residues, and 1 COOH-terminal leucine residue) are all on the surface of the molecule. Four of the carboxyl residues are in the cleft region in which the substrate is bound and two of the residues, aspartic 52 and glutamic 35, have been postulated to be directly involved in the catalytic action of the enzyme.

Recently in our laboratory a carboxyl group modification procedure (4-6) has been developed which is capable of specifically changing the carboxyl groups of a protein without altering other amino acid residues. It seemed desirable to treat lysozyme with this reagent in order (a) to correlate rates of modification with the structural features of the protein and (b) to test the hypothesized catalytic roles of glutamic 35 and aspartic 52 residues. The preliminary results of such studies are reported here.

The lysozyme (crystalline Armour enzyme) at a concentration of 5 to 10 mg per ml was treated at pH 4.75 and 25°C following the procedure previously described (5). The carbodiimides used were 1-ethyl-3-dimethylaminopropyl carbodiimide hydrochloride or 1-benzyl-3-dimethylaminopropyl carbodiimide tosylate at initial concentrations of 0.1 M. The nucleophiles used were 0.25 M aminomethanesulfonic acid (NH₂CH₂SO₃H), 1.0 M glycine methyl ester, or 1.0 M glycinamide. Substitution by NH₂CH₂SO₃H changes the bulk but not the net charge of the protein whereas Gly-OMe and Gly-NH₂ incorporation changes both size and charge. When the sulfonic acid was used, 0.7 M NaCl was added to the medium. Enzyme activity was determined by Micrococcus lysodeikticus cell lysis at pH 7.0 in phosphate buffer (7). Incorporation was determined by the chromotropic acid reagent (8), by using tritium or C-labeled NH₂CH₂SO₃H, or by measuring the remaining free carboxyl groups by subsequent treatment with the carbodiimide reagent and Gly-OMe in the presence of 4 M guanidine. Glycine ester or amide incorporation was determined on the amino acid analyzer (9) or by using radioactive glycine derivatives. Protein was estimated from its absorbance at 280 mp from a molar extinction coefficient for native enzyme (mol wt 14,500) of 3.9 x 10⁴ in water or in 0.2 M acetate buffer at pH 4.75. The corresponding value for protein modified with 10 NH₂CH₂SO₃H groups was 3.8 x 10⁴.

With NH₂CH₂SO₃H, Gly-OMe, or Gly-NH₂, all 11 carboxyl side chains of hen's egg white lysozyme were quantitatively modified with carbodiimide reagent in a high concentration of guanidine (>3.5 M). The protein modified in this manner was inert toward cell walls of M. lysodeikticus and toward N-acetylmuramylpentaglycine oligosaccharide. In the absence of guanidine, the extent of carboxyl modification tended to level off after 200 min and the number of modified carboxyl groups reached a limiting value which depended somewhat on the nucleophile in the reaction (cf. Fig. 1). About 8.5 to 9.5 sulfonate groups or 6.5 to 7.5 Gly-OMe groups were incorporated per molecule of protein depending on the conditions. This difference is intriguing since it must reflect significant influences of the environmental effects in the two reactions. Clearly at least 10 residues must be activated by the carbodiimide reagent, since both steps require the same first step activation. Yet 2 of these activated residues which react with NH₂CH₂SO₃H are inaccessible or sluggish to subsequent nucleophile attack by the Gly OMe molecule even after long intervals. In other studies to be reported the rate of carboxyl activation also varies for different carboxyl groups.

When N-acetylmuramylpentaglycine trimer and tetramer were included in the reaction mixture, the initial rapid loss in the enzyme activity was prevented and about 50% of the native activity was retained after reaction for 400 min with NH₂CH₂SO₃H or Gly-OMe as the nucleophilic reagents (cf. Fig. 1). As in the unprotected reaction, the number of carboxyls modified also converged to the limiting values of 7 to 8 sulfonate groups and 5.5 to 6.5 Gly-OMe groups, respectively.

By increasing the amount of N-acetylmuramylpentaglycine oligosaccharide, the extent of protection increased to a limiting value. N-Acetylmuramylpentaglycine monomer was a relatively poor protector.

In previous reports, Dahlquist, Jao, and Raftery (10), Rupley et al. (11), and Chipman, Grisaro, and Sharon (12) have shown the trimer, tetramer, and pentamer derivatives to have approximately the same affinity toward the enzyme. The better pro-

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The abbreviations used are: Gly-OMe, glycine methyl ester; Gly-NH₂, glycaminde.
tection of the higher oligomers resulted presumably from a more efficient masking of the catalytic center with the larger molecules. Cellobiose, which can be an acceptor in transglycosylation, does not protect the enzyme. The specificity of the protection reaction is summarized in Table I.

In order to clarify the reactivity and roles of specific carboxyl groups the following experiment was performed. Lysozyme (100 mg) was modified in the presence of a trimmer-tetramer-pentamer substrate mixture (15 mg per ml) (designated as +S) and in its absence (designated -S) for 400 min by using unlabeled NH₂CH₂SO₃H. The sample containing substrate retained 66.6% of its activity and had 7.3 carboxyl residues modified. The protein in the -S experiment had 8.4 residues modified and was enzymatically inactive (less than 1%). At this point both samples were dialyzed (to remove the protecting substrate in the +S experiment and for control purposes in the -S experiment) and then treated for 300 min with ¹⁴C-labeled NH₂CH₂SO₃H. The sample containing substrate retained 56.5% of its activity and had 7.3 carboxyl residues modified. The protein in the -S experiment incorporated 0.5 additional groups. After dialysis the proteins were 

<table>
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<th>Saccharide</th>
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<th>Residual activity</th>
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</table>

**TABLE II**

Modification of carboxyl groups of lysozyme by NH₂CH₂SO₃H in presence and absence of substrate

The general procedure of the experiment is described in the text. Detailed conditions were as follows: 0.1 M 1-ethyl-3-dimethylaminopropyl carbodiimide hydrochloride, 0.25 M NH₂CH₂SO₃H, 0.7 M NaCl, 25°C, pH 4.75. Step 1 unlabelled NH₂CH₂SO₃H; Step 2 after removal of S, ¹⁴C-labeled NH₂CH₂SO₃H; Step 3 in 4 M guanidine, ¹⁴C-labeled NH₂CH₂SO₃H.

*The T₈ peptide also contains aspartic 48. Edman degradation results indicated that essentially all of the radioactivity was in aspartic 52. Therefore, aspartic 48 was essentially completely modified in Step 1 both in the presence and absence of substrate. No attempt was made to determine which residue contained radioactive activity in this peptide since the amounts were so small.*

Because glutamic 35 was not modified to a significant extent in these studies, identification with activity loss is less strong than that of aspartic 52. The x-ray crystallography studies place it immediately adjacent to aspartic 52, however, and a protection experiment therefore place it near the site of catalytic activity in an independent investigation.

M. Parsons and M. Raftery, personal communication.
tion with glutamic 35. The behavior in the carboxyl modification studies would suggest that the glutamic 35 group is the carboxyl residue which was shown by Donovan, Laskowski, and Sheraga (17) to have an abnormal pK value.

These results do not establish the precise roles of glutamic 35 or aspartic 52 but they do provide strong support for the hypothesis of Phillips et al. (2, 3) that the groups are involved in catalytic activity since they (a) eliminate all other carboxyl groups as essential catalytic residues, (b) are protected by substrate, and (c) are modified with concomitant loss of activity.

In addition to the information obtained about the catalytic groups, these studies provide structural information which supports and complements the crystallographic findings. In the first place they are consistent with the crystallographic finding that essentially all of the carboxyl groups are on the surface of the molecule. However, the fact that some of these surface groups are not quantitatively modified suggests that the reactivities depend on the specific nature of these individual environments, e.g. the notable difference in reactivities in the displacement of the carbodiimide by a nucleophile. An intriguing possibility for the difference between the Gly-OMe and NH₂CH₂SO₃H results is that the protein is acquiring a positive charge in Gly-OMe modification whereas the charge remains the same in the NH₂CH₂SO₃H modification. A further interesting feature is the influence of substrate on the reactivities of some residues which are not in the cleft or in direct contact with substrate, e.g. aspartic 87, aspartic 66, and glutamic 7 (cf. Table II). This suggests that conformational changes are occurring under the influence of substrate. Blake et al. (2) and Phillips (3) have detected a movement of the tryptophan residue induced by inhibitors. The present studies give the suggestion that in solution effects may be occurring.

The modification of aspartic 101 is interesting since this group has been postulated to be a binding group in hydrogen bond contact with the saccharide in the lysozyme-tri-N-acetylglucosamine complex. Apparently the saccharides provide little protection for modification of this aspartic 101 and moreover its modification does not eliminate enzyme activity against cell walls.

The carboxyl modification technique makes it possible to modify groups in ways other than the introduction of the NH₂CH₂SO₃H, Gly-OMe, or Gly-NH₂ derivatives. For example, it has been shown that carboxyl groups can be converted to amino groups (15). The finding that all of the carboxyl residues except glutamic 35 and aspartic 52 can be blocked with retention of enzyme activity makes it possible to prepare other modifications of aspartic 52 and glutamic 35 which may throw further light on their specific function. These studies are in progress.

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