# MODIFICATION OF CHYMOTRYPSINOGEN WITH 1-AMINOGLUCOSE

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## 1. Introduction

In connection with our interest in determining whether carbohydrate residues help determine the half-life of certain asparaginases introduced into the circulation, we have sought methods for attaching carbohydrates to proteins by chemically defined linkages. Water-soluble carbodiimides were first used to modify protein carboxyl groups by Sheehan and Hlavka [1], but the reaction has been developed more fully by Koshland and his colleagues [2]. Since carbodiimide coupling leads to the formation of amides by condensation of amino compounds with carboxyl groups, it appeared that this reaction might be used to introduce suitable sugar derivatives into proteins. The use of 1-aminoglucose to form glucosyl derivatives of chymotrypsinogen is described here.

## 2. Materials and methods

D-1-aminoglucose was synthesized by the method of Isbell and Frush [3] and the purity of the product checked by elemental analysis. Alpha-chymotrypsinogen A (bovine, 6 X crystallized, Type II) was obtained from Sigma, and trypsin (porcine, crystalline) from Novo Industri, A/S, Copenhagen. After rapid activation at pH 7.5, chymotryptic activity was determined by the procedure of Hummel [4]. Since the modification reaction to be described affects the rate of tryptic activation, assays were routinely performed on samples allowed to stand with trypsin for both 2 and 6 hr. The substrate, benzoyl-L-tyrosine ethyl ester, was obtained from Aldrich Chemical Co. Chymotrypsinogen concentration was estimated from its absorbancy at 280 nm using 0.49 as the molar extinction coefficient. Glucose was determined by the glucose oxidase kinetic method [5], using reagents provided by AB Kabi, Stockholm, and the LKB 8600 reaction rate analyzer. Glucose was released from modified chymotrypsinogen by hydrolysis in 2 N HCl for 1 hr at 110° in vacuo. Samples were dried in vacuo at room temp., and then taken up in water.

Free carboxyl groups in modified chymotrypsinogen were determined by the method of Hoare and Koshland [6] using norleucine methylester as the nucleophile. D,L-Norleucine methyl ester HCl and 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide (EDC) were obtained from Sigma. Free amino groups were determined by carbamylation [7] followed by determination of homocitrulline on the amino acid analyzer. Amino acid analyses were performed according to Spackman et al. [8] on a Beckman/Spinco model 120 amino acid analyzer modified to an accelerated system requiring 3.5 hr for a complete analysis. When homocitrulline was to be determined, the pH of the first buffer was increased from 3.28 to 3.31, giving satisfactory separation of the homocitrulline peak just preceding valine [9]. No changes were needed for norleucine, which emerges just after leucine. Homocitrulline values have been corrected upwards by 12.5%, and lysine values downwards by the same amount, to allow for homocitrulline hydrolysis.

## 3. Results and discussion

For the coupling of 1-aminoglucose, chymotrypsinogen was dissolved in water (10 mg/ml), D-1-aminoglucose added (0.5 M), and the pH adjusted to 5.0 with 1 N HCl. Solid EDC was then added (to 0.1 M), the pH adjusted again if necessary, and maintained at

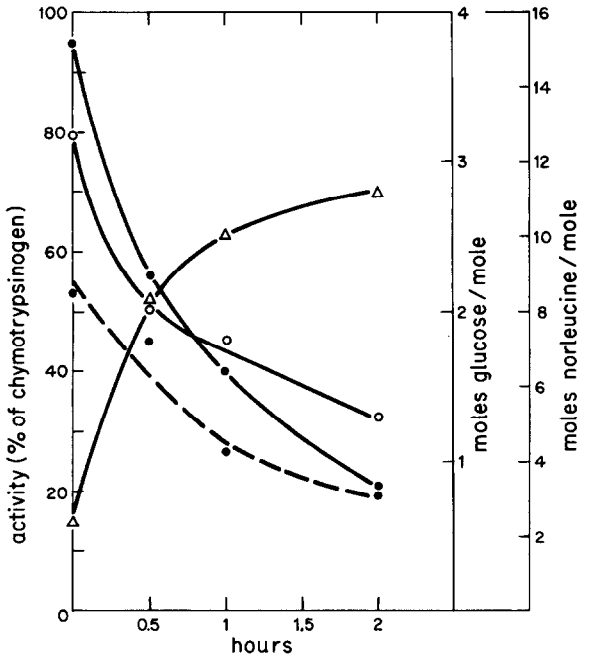


Fig. 1. Coupling of 1-aminoglucose to chymotrypsinogen. Filled circles (•) with dashed line (---) and solid line (——) show activity after 2 and 6 hr activation, respectively; open circles (○) show moles norleucine/mole modified enzyme, equivalent to free carboxyl groups; and triangles (△) show moles of glucose/mole.

pH 5.0 with the pH stat. A "zero-time" sample was usually withdrawn as soon as the pH was adjusted after EDC addition. Samples were quenched in 1 M acetate buffer, pH 5.0, and dialyzed against 0.001 N HCl in the cold. Control experiments showed that quenching had little or no effect on the parameters measured. In some experiments, more 1-aminoglucose and/or EDC was added.

The results of a typical experiment are shown in fig. 1. At 1 hr about 2.5 residues of glucose are bound, on the average, per mole of chymotrypsinogen, with the retention of about 40% activity. Prolonging the reaction to 2 hr and adding more EDC and 1-aminoglucose only increased the number of glucose residues to 2.8, with a decline in activity. A systematic attempt to establish maximal conditions has not yet been made, but table 1 shows the effect of pH, initial concentration of 1-aminoglucose, etc., on the reaction.

Many proteins have been coupled to agarose, cellulose and the like by methods involving activation of

Table 1
Effect of certain variables on the coupling of 1-aminoglucose to chymotrypsinogen.

| Sample | Activity as % of chymotrypsinogen) | (Moles glucose/mole enzyme) | Free COOH groups/mole modified enzyme) |
|---|---|---|---|
| Effect of pH: | | | |
| pH 5 | 24 | 2.8 | 6.1 |
| pH 6 | 36 | 1.3 | 10.0 |
| pH 7 | 51 | 1.0 | 12.1 |
| Effect of conc. of 1-aminoglucose | | | |
| 1 M | 38 | 1.9 | 6.7 |
| 0.5 M | 39 | 1.5 | 8.1 |
| 0.1 M | 21 | 0.8 | 6.2 |
| Effect of more EDC | | | |
| EDC at start only | 40 | 2.5 | 7.3 |
| EDC also added at 30 min | 45 | 3.3 | 7.6 |

All incubations carried out for 1 hr at pH 5 at room temp. with 0.5 M 1-aminoglucose unless otherwise specified. Activity determined after 6 hr activation. Free carboxyl groups are equivalent to moles of norleucine taken up by the modified enzyme (theoretically 15 with unmodified chymotrypsinogen, obtained 14.4).

the polysaccharide [10]. The linkages produced are not like those found in native glycoproteins, however, and the methods do not lend themselves readily to the introduction of simple sugar derivatives into proteins. In the reaction with 1-aminoglucose reported here, EDC-activated carboxyl groups of aspartic and glutamic acid form N-glycosyl derivatives which, in the case of the aspartyl group, are indistinguishable from the carbohydrate-asparagine linkage found in many glycoproteins, except that presumbaly a mixture of alpha- and beta-glycosides would be present. The properties of the modified enzyme are due in part to side-reactions (see below) but the use of a specific enzyme assay establishes unequivocally that glucosyl residues have been introduced.

Carraway et al. [11] and Abita et al. [12] on the one hand, and Fersht and Sperling [13] on the other,

Table 2
Side-reactions in the coupling of 1-aminoglucose to chymotrypsinogen.

| Sample | Activity (as % of chymotrypsinogen) | (Free COOH groups/mole of modified enzyme) |
|---|---|---|
| 0-time | 91 | 12.2 |
| 1/2-hr | 32 | 9.9 |
| 1 hr | 19 | 6.4 |
| 2 hr | 8 | 4.5 |

Incubated with EDC alone at pH 5 at room temp., activity determined after 6 hr activation. Free carboxyl groups are equivalent to moles of norleucine taken up by the modified enzyme.

used carbodiimide coupling to block most of the carboxyl groups of chymotrypsinogen with glycine methyl ester and semicarbazide, respectively, and showed that Asp-194 and Asp-102, known to be buried on the basis of X-ray studies, are not modified. The fact that fewer residues of glucose (about 3) are introduced here than of glycine methyl ester or semicarbazide (about 11 in both cases) under similar conditions may be due partly to the fact that 1-aminoglucose is a larger molecule, and may not have completely free access to all "surface" carboxyl groups.

There are 15 carboxyl groups in chymotrypsinogen, and thus 12 norleucines should be taken up during coupling with modified enzyme containing 3 glucose residues. The fact that such results are not obtained (fig. 1 and table 1) suggests that side-reactions are occurring, and these may be responsible for much of the loss of activity. Riehm and Scheraga [14] have shown that ribonuclease is extensively modified by water-soluble carbodiimide alone, but the precise nature of the reactions taking place was not established. Ribonuclease activity declined as the number of additions of water-soluble carbodiimide increased. Here also, in the presence of EDC alone, both activity and free carboxyl groups decline with time (table 2). The activity of chymotrypsinogen exposed to EDC alone is only about half that found in the presence of EDC and 0.5 M 1-aminoglucose. Rearrangement to an *N*-acyl urea of the initial *O*-acyl adduct formed between carboxyl groups and EDC seems to be the most likely side-reaction, but cross-linking probably occurs also, since carbamylation shows that epsilon-amino groups of lysine

Table 3
Side-reactions in the coupling of 1-aminoglucose to chymotrypsinogen.

| Sample | Free $\epsilon$-$NH_2$ groups/mole of modified chymotrypsinogen | Unreactive $\epsilon$-$NH_2$ groups/mole of modified chymotrypsinogen |
|---|---|---|
| 1 M 1-aminoglucose | 10.6 | 2.5 |
| 0.5 M 1-aminoglucose | 9.7 | 3.6 |
| 0.1 M 1-aminoglucose | 8.7 | 4.4 |

Incubated at pH 5 for 1 hr at room temp.; activity determined after 6 hr activation. Free $\epsilon$-amino groups are equivalent to moles of homocitrulline formed upon carbamylation of the modified enzyme, and unreactive $\epsilon$-amino groups are equivalent to lysine.

also disappear in coupling experiments with 1-aminoglucose (table 3).

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