Chemical modification of *Penicillium* 1,2- α -D-mannosidase by water-soluble carbodi-imide: identification of a catalytically important aspartic acid residue

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1,2- α -D-Mannosidase from *Penicillium citrinum* was inactivated by chemical modification with 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC). Most of the activity was lost after modification in the absence of a nucleophile, glycine ethyl ester. 1-Deoxymannojirimycin (dMM), a competitive inhibitor of the enzyme, showed partial protection against the inactivation. After the modification by EDC without the presence of a nucleophile, proteolytic digests of the enzyme were analysed by reversed-phase h.p.l.c., and a unique peptide was shown to decrease when dMM was present during the modification. The peptide was absent from the digests of unmodified enzyme. The amino acid

sequence of the peptide (A; Ile-Gly-Pro) was identical in part with that of the adjacent peptide (B; Ile-Gly-Pro-Asp-Ser-Trp-Gly-Trp-Asp-Pro-Lys). When cholecystokinin tetrapeptide (Trp-Met-Asp-Phe-NH₂) was modified by EDC alone, the modified peptide could be separated from unmodified peptide by reversed-phase h.p.l.c., and Edman degradation was stopped before the modified aspartic acid residue. This suggested that, in the enzyme, peptide A was derived from peptide B by the modification. Consequently, Asp-4 in peptide B was assumed to be masked by dMM during the modification, and to be involved in the interaction of the enzyme with its substrate.

INTRODUCTION

Chemical modification of amino acids provides a useful way of studying protein structure as well as altering function. Water-soluble carbodi-imide has previously been employed in chemical modification of carboxy groups in acidic amino acids [1]. The modification basically consists of two reactions: the activation of a carboxy group by a water-soluble carbodi-imide to form an *O*-acylisourea structure, which in turn reacts with a nucleophile such as glycine ethyl ester (GEE) to form an amide bond [1]. Because of its high solubility in water, the rather mild conditions required for the modification and the relatively high specificity towards acidic amino acids, water-soluble carbodi-imide has often been used to modify native enzymes presumed to have carboxy groups at their catalytic sites. In egg white lysozyme, approx. 8–9 (there is a total of 10 in a molecule) carboxy groups were modified by this method [2]. In the experiment, the presence of an *N*-acetylglucosamine oligomer protected Asp-52 from modification, indicating that the residue is catalytically essential [2]. In many studies, modification by a water-soluble carbodi-imide has been performed in the presence of appropriate nucleophiles. However, RNAase A [3], rabbit-muscle phosphorylase *b* [4] and *Rhizopus* glucoamylase [5] could be inactivated by reaction with water-soluble carbodi-imide in the absence of nucleophiles. These experiments suggest that, in some cases, an intermediate form during the carbodi-imide reaction can be fixed without appropriate nucleophilic reagents.

Some glycan-hydrolysing enzymes such as Taka-amylase A [6], soyabean β -amylase [7] and almond β -glucosidase [8] are known to have essential carboxy groups in the catalytic domain. We have previously purified and characterized several 1,2- α -mannosidases (mannosyl-oligosaccharide 1,2- α -mannosidase; EC 3.2.1.113) from moulds [9–12]. These enzymes are highly

specific toward 1,2- α -mannosidic linkages and were utilized in analysis of the glycan structure [13,14]. One of them, the enzyme from *Penicillium citrinum*, is a monomer with *M_r* 53000, and shows maximal activity at pH 5.0 [12]. Unlike the other glycanases mentioned above, the structure of the catalytic domain of α -mannosidases has never been well documented. Here, we describe the chemical modification of *Penicillium* 1,2- α -mannosidase using 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC), together with the deduced structures of derivative peptides observed.

MATERIALS AND METHODS

Materials

EDC and GEE were purchased from Nacalai Tesque, Kyoto, Japan. *Penicillium* 1,2- α -D-mannosidase Ib was prepared as described previously [12] using a commercial product, 'Protease B' (generously donated by Amano Pharmaceutical Co., Nagoya, Japan) as a starting material. From 30 g of Protease B, approx. 0.8 mg of purified 1,2- α -D-mannosidase Ib, which had a specific activity of 7.6×10^{-3} kat/kg toward yeast mannan, was obtained. The enzyme was exhaustively dialysed against distilled water; the cellulose tube was then hung near a ventilator in a cold-room. In this way we could prepare an approx. 5-fold-concentrated enzymic solution free of acetate ions. Baker's yeast mannan, 1-deoxymannojirimycin (dMM) and Man α 1 $\rightarrow$ 2Man-OMe were purchased from Sigma, St. Louis, MO, U.S.A. Lysylendopeptidase (*Achromobacter* protease I; EC 3.4.21.50 [15]) was from Wako Pure Chemical Industries, Osaka, Japan. Cholecystokinin (CCK) tetrapeptide (Trp-Met-Asp-Phe-NH₂) and neurokinin A (His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH₂) were purchased from Peptide Institute, Osaka, Japan.

Modification of 1,2- α -D-mannosidase for kinetic analysis

Chemical modifications were carried out in 100 μ l of solutions containing 100 mM *NNN'*-tetramethylethylenediamine (TEMED)/HCl, pH 4.7, 3.6 μ g of enzyme, 250 mM GEE and EDC (5–100 mM) at 30 °C for a predetermined period of time. The reactions were stopped by adding 10 μ l of 1 M sodium acetate/acetic acid buffer, pH 5.0, and the solutions were dialysed against 10 mM sodium acetate/acetic acid buffer, pH 5.0, at 4 °C. The dialysis was performed exhaustively because remaining EDC gave a large blank value in the following assay. Small samples (45 μ l) of the dialysed solutions were used to determine residual activity.

When the substrates and inhibitor were examined for protective effects, enzyme modifications were performed with 50 mM EDC and 250 mM GEE in 110 μ l of 100 mM TEMED/HCl buffer, pH 4.7, at 30 °C for 20 min in the presence of protective reagent. In each case, the enzyme and reagent had been incubated at 30 °C for 5 min before the modification.

Enzyme assays and protein determination

1,2- α -D-Mannosidase activity was determined by a published method [12]. Basically, Man α 1-2Man-OME was hydrolysed by the enzyme at 30 °C in 50 mM sodium acetate/acetic acid buffer, pH 5.0, and released mannose was determined by the method of Park and Johnson [16]. One katal of 1,2- α -D-mannosidase is defined as the amount of enzyme required to liberate 1 mol of mannose/s at 30 °C and pH 5.0. When α -mannan (which could not be removed by dialysis) was used as the protecting reagent during the modification, residual activity of the enzyme was measured using pyridylaminated high-mannose-type oligosaccharide (Takara Shuzo Co., Kyoto, Japan) as a substrate, for which h.p.l.c. analysis was performed as described by Hase *et al.* [17]. Protein concentrations were measured by the method of Lowry *et al.* [18], with BSA as standard.

Modification of 1,2- α -D-mannosidase by EDC and *N*-(1-naphthyl)ethylenediamine (EDAN)

Penicillium 1,2- α -mannosidase (44 μ g; 0.83 nmol) was incubated in 300 μ l of solution containing 100 mM TEMED/HCl, pH 4.7, 50 mM EDC and 20 mM EDAN at 30 °C for 30 min in the presence or absence of 5 mM dMM. In this case the enzyme and dMM were incubated at 30 °C for 5 min before EDC and EDAN were added. The modification reaction was stopped by the addition of 30 μ l of 1 M sodium acetate/acetic acid buffer, pH 5.0, and the solution was dialysed against 1 litre of 20 mM sodium acetate/acetic acid buffer, pH 5.0 at 4 °C for 4 days during which time the buffer was changed four times. Then fluorescence intensity of the internal solution was measured at an excitation wavelength of 330 nm and emission wavelength 430 nm using a fluorescence spectrophotometer (Hitachi, model F-3000).

Modification of 1,2- α -D-mannosidase for peptide analysis

For the identification of modified amino acids, 160 μ g of *Penicillium* 1,2- α -mannosidase was incubated in 600 μ l of solution containing 100 mM TEMED/HCl, pH 4.7, and 50 mM EDC at 30 °C for 60 min in the presence or absence of 4.1 mM dMM. In this case the enzyme and dMM were incubated at 30 °C for 5 min before the addition of EDC. The modification reaction was stopped by adding 60 μ l of 1 M sodium acetate/acetic acid buffer, pH 5.0, and the solution was dialysed against 1 litre of 20 mM sodium acetate/acetic acid buffer, pH 5.0, at 4 °C for 2

days, with two changes of buffer. The cellulose tube was then soaked in 100 ml of 8 M urea solution and dialysed overnight at room temperature. Denatured enzyme was recovered, diluted with 1 vol. of distilled water to 4 M urea concentration, and concentrated using a Centricon Super Mini W-10 (Kurabo Co., Osaka, Japan), a centrifugal microconcentrator that cuts off molecules below M_r 10000. To the concentrated enzyme (approx. 0.3 ml) were added 15 μ l of 1 M Tris/HCl buffer, pH 8.0, and 1 μ l of lysylendopeptidase solution (5 mg/ml), and digestion was carried out at 30 °C for 3 days. The mixture was then filtered using Centricon Super Mini W-10 and the filtrate was analysed by reversed-phase h.p.l.c.

Modification of short peptides

CCK tetrapeptide (1 mg) was dissolved in a solution (1 ml) containing 100 mM TEMED/HCl, pH 4.7, and 1 M guanidinium chloride. Then EDC (powder) was added to 100 mM, followed by incubation at 30 °C for 1 h. The solution was passed through a short column of octylsilyl silica gel (Lichrolut RP-select B; Merck; 0.5 g of gel in a column), which was then washed in 10 % acetonitrile/0.1 % trifluoroacetic acid (TFA). Peptides were eluted in 99.9 % acetonitrile/0.1 % TFA, and then dried using a centrifugal concentrator. The dried peptides were dissolved in water or 100 mM Tris/HCl, pH 8.0, each containing 2 M guanidinium chloride, and kept at 30 °C overnight before reversed-phase h.p.l.c. analysis. Modification of neurokinin A was performed in the same way except without guanidinium chloride. To examine whether dMM could act as a nucleophile, CCK tetrapeptide (41 μ g) was modified in a solution (100 μ l) containing 50 mM EDC, 100 mM TEMED/HCl, pH 4.7, and 1 M guanidinium chloride in the presence or absence of 10 mM dMM at 30 °C for 1 h. The reaction products were prepared for reversed-phase h.p.l.c. as described above.

Reversed-phase h.p.l.c. and amino acid sequencing

Reversed-phase h.p.l.c. was performed using a Lichrospher 100 CN (5 μ m) column (250 mm $\times$ 4 mm) with a gradient of acetonitrile (0.8, 8 and 64 % at 0, 1 and 80 min respectively) in 0.1 % TFA at a flow rate of 0.6 ml/min. Eluates were monitored at 215 nm. Amino acid sequence was determined using an Applied Biosystems (ABi) 473A gas-liquid-solid-phase protein sequenator with an ABi 610A data-analysis system.

Amino acid analysis

Dried preparations of peptides were hydrolysed in 4 % thioglycolic acid and 6 M HCl at 110 °C for 24 h. Peptide hydrolysates were analysed on an amino acid analyser (Hitachi; model L-8500).

M.s.

M.s. determination of peptides was performed by the electrospray ionization method [19,20]. Peptides dissolved in 50 % methanol/water were injected into the electrospray chamber at 0.2 ml/min, and analysed on a mass spectrometer (Finnigan Mat; model TSQ 700). Ionization was achieved at 4.5 kV.

RESULTS

Effects of EDC concentration on inactivation of 1,2- α -D-mannosidase

We used EDC for enzyme modification because of its high solubility in water. *Penicillium* 1,2- α -D-mannosidase seemed to

Figure 1 Inactivation of *Penicillium* 1,2- α -D-mannosidase by EDC and GEE

The enzyme was modified with 5 (○), 25 (●), 75 (□) and 100 (■) mM EDC in the presence of 250 mM GEE at 30 °C, then residual activity was measured as described in the Materials and methods section. Control solutions without EDC were also incubated and dialysed in parallel with the modified enzymes. Modification experiments were performed in duplicate and the enzyme activities given are the means.

Figure 2 Inhibition of *Penicillium* 1,2- α -D-mannosidase by dMM

The reaction mixture (50 μ l) contained approx. 0.3 μ g of enzyme, various concentrations of dMM and 1.12 (●), 0.56 (○), or 0.34 (■) mM Man α 1-2Man-OMe in 50 mM sodium acetate/acetic acid, pH 5.0. Reactions were carried out at 30 °C for 5 min and enzyme activities given are the means of duplicate assays. Dixon plots are shown.

be suitable for modification because it is stable at pH 4–5, and, under these conditions, EDC modifies proteins most effectively [21]. When the effects of EDC concentration were examined, the inactivation rate was found to increase as the concentration of EDC increased (Figure 1).

Effects of substrates and inhibitor on EDC inactivation

If EDC inactivation is retarded by the presence of substrates or their analogues during the reaction, the catalytically important amino acid in *Penicillium* 1,2- α -mannosidase can be assumed to be protected from modification. We examined various substrates for their ability to protect against inactivation. The presence of yeast α -mannan (136 mg/ml) during the modification process

Table 1 Effect of dMM on inactivation of *Penicillium* 1,2- α -D-mannosidase by EDC

Enzyme (1.8 μ g) was incubated with various concentrations of dMM (0.1–3.5 mM) in 100 μ l of 100 mM TEMED/HCl buffer, pH 4.7, at 30 °C for 20 min. Then EDC and GEE were added to 100 mM and 250 mM respectively and the mixture was further incubated at 30 °C for 20 min. The modification was stopped by adding 15 μ l of 1 M sodium acetate buffer, pH 5.0, and residual activity was measured as described in the Materials and methods section. Data are expressed as means of duplicate assays.

	[dMM] (mM)	Activity (pkat)	Relative activity (%)
Intact enzyme	0	91.7	100
Modified enzymes	0	11.3	12.3
	0.1	12.0	13.1
	0.5	22.6	24.6
	1.0	30.5	33.3
	1.5	30.2	33.0
	3.5	31.5	34.3

did not provide protection. When Man α 1-2Man-OMe (4.34 mM) was added to the EDC-containing reaction mixture, a little activity (approx. 10%) remained after the modification. The presence of 10, 50, 100 or 300 mM α -methylmannose gave weak protection (5, 7, 10 or 16% of native enzyme activity remained). dMM is a substrate analogue designed to inhibit mammalian Golgi mannosidase IA [22]. This compound showed competitive inhibition toward *Penicillium* 1,2- α -mannosidase with a K_i value of 105 μ M (Figure 2). When modification was performed in the presence of dMM, the enzyme was partially protected from inactivation (Table 1). The increase in protection was not linear with dMM concentration above 1.0 mM, and 34% of activity was protected from inactivation in the presence of 3.5 mM dMM. Of all the compounds tested, dMM provided the maximum protection.

Effect of nucleophilic reagents on modification

To investigate the effects of nucleophilic reagents on modification, the enzyme was modified by EDC in the presence or absence of GEE and the degrees of inactivation were compared (Figure 3). *Penicillium* 1,2- α -mannosidase was found to be modified and inactivated by EDC in the absence of nucleophilic reagents. Approximately 14–20% more inactivation occurred in the presence of GEE, whereas dMM showed a similar extent of protection under both conditions.

We then attempted to label carboxy groups in the enzyme using a fluorescent reagent, EDAN, as a nucleophile instead of GEE during the modification reaction. EDAN was used in uronic acid [23]. Preliminarily, a couple of enzyme samples were modified by 25 mM EDC, one in the presence of 40 mM EDAN and the other in the presence of 250 mM GEE, and the residual activities were monitored. The two enzyme samples lost activity at the same rate (both showed approx. 63, 45 and 25% of initial activity after 10, 20 and 45 min of modification respectively) meaning that EDAN is as effective a nucleophile in the amidation reaction as GEE. The enzyme samples were then modified with 50 mM EDC and 20 mM EDAN for 30 min in the presence or absence of dMM, and EDAN incorporation was compared. Both (approx. 0.8 nmol of protein) showed the same fluorescence intensity which was equivalent to 0.7 nmol of EDAN. When the conditions of modification were more severe (100 mM EDC and 20 mM EDAN for 60 min), the enzyme samples showed the same fluorescence intensity, again irrespective of the presence of dMM

Figure 3 Inactivation of *Penicillium* 1,2- α -D-mannosidase by EDC in the presence or absence of GEE

The enzyme (approx. 3 μ g) was incubated in 100 μ l of solution containing 100 mM TEMED/HCl, pH 4.7, and 50 mM EDC with (●, ○) or without (■, □) 250 mM GEE at 30 °C for the periods of time indicated in the presence (○, □) or absence (●, ■) of dMM (4 mM). After the reaction was stopped by the addition of 10 μ l of 1 M sodium acetate buffer, pH 5.0, and chilling in an iced bath, each solution was dialysed against 1 litre of 50 mM sodium acetate buffer, pH 5.0, overnight at 4 °C. The residual activity was measured as described in the Materials and methods section. Assays were performed in duplicate and the mean values are given.

(approx. 0.8 nmol of EDAN was incorporated into 0.8 nmol of protein under both conditions). Whereas dMM partially protected the enzyme activity, it did not affect incorporation of EDAN. These findings suggest that the enzyme may have a carboxy group that can be masked by dMM but does not incorporate nucleophiles.

Isolation of the modified peptide

Penicillium 1,2- α -mannosidase was modified by EDC without nucleophilic reagents in the presence or absence of dMM, then digested with lysylendopeptidase (*Achromobacter* protease I) at pH 8 after denaturation, and peptides were fractionated by reversed-phase h.p.l.c. A few peaks changed in height in response to the presence of dMM during the modification (Figure 4). Peak A was unique in that it increased in the absence of dMM, whereas peak B was larger in the presence of dMM. This observation was reproduced when the modification reaction was carried out for 10, 30 or 120 min, both in the presence or absence of dMM (results not shown). The following sequences were obtained by Edman degradation: peptide A, Ile-Gly-Pro; peptide B, Ile-Gly-Pro-Asp-Ser-Trp-Gly-Trp-Asp-Pro-Lys. Peptide B had a sequence identical with peptide A within it. In the routine sequencing of an amino acid, the C-terminal residue can often be seen as a repetition of the same amino acid decreasing with cycles, followed by the absence of any peaks. In the analysis of peptide A, no peaks were detectable after the fourth cycle of sequencing, meaning that either the C-terminal amino acid residue was proline or sequencing was interrupted. The peak representing peptide A was not observed on reversed-phase h.p.l.c. of lysylendopeptidase digests of unmodified enzyme. It did not seem likely that peptide A resulted from proteolytic cleavage of another amino acid sequence such as -Ile-Gly-Pro-X-, somewhere within the polypeptide. These results suggest that

Figure 4 Reversed-phase h.p.l.c. profiles of modified 1,2- α -D-mannosidase digested by lysylendopeptidase

Penicillium 1,2- α -mannosidase was incubated in a solution containing 100 mM TEMED/HCl, pH 4.7, and 50 mM EDC in the absence (a) or presence (b) of 4.1 mM dMM for 60 min at 30 °C. The modified enzyme was denatured in 8 M urea, then diluted and digested by lysylendopeptidase in 4 M urea and 50 mM Tris/HCl, pH 8.0, at 30 °C. The solution was analysed by reversed-phase h.p.l.c. (Lichrospher 100; CN column). Peptides were eluted in an increasing gradient of acetonitrile in 0.1% TFA, and monitored at 215 nm. Experimental details are given in the Materials and methods section.

amino acid sequencing of peptide A was interrupted before Asp-4, probably as a result of the modification.

Modification of a short peptide

Although Asp-4 was proposed to be modified in peptide A, it was not certain that a peptide modified by EDC, without nucleophilic reagents, could be distinguished from intact peptide on reversed-phase h.p.l.c., and that amino acid sequencing was interrupted before the modified amino acid residue. To clarify these uncertainties, two short peptides were modified. First, CCK tetrapeptide (Trp-Met-Asp-Phe-NH₂) was modified by EDC alone and analysed by reversed-phase h.p.l.c. (Figure 5). When the modified peptide, which had been dissolved in 2 M guanidinium chloride solution and left intact (pH 2.6) was analysed, a peak of R_t 34 min (II) appeared as a major product after the unmodified peptide (I) (Figure 5a). However, a dramatic change was caused by raising the pH of the peptide solution. When the modified peptides were dissolved in alkaline solution (100 mM Tris/HCl, pH 8.0) containing 2 M guanidinium chloride before loading on to the h.p.l.c. column, peak II decreased and a new peak appeared at R_t 24 min (peak III in Figure 5b). The following

Figure 5 Reversed phase h.p.l.c. profiles of CCK tetrapeptide modified by EDC

CCK tetrapeptide (Trp-Met-Asp-Phe-NH₂) was incubated in a solution containing 100 mM TEMED/HCl, pH 4.7, 1 M guanidinium chloride and 100 mM EDC for 1 h at 30 °C. After partial purification, modified peptide (dried pellet) was dissolved in water (a) or in 100 mM Tris/HCl, pH 8.0 (b), then analysed by reversed-phase h.p.l.c. (Lichrospher 100; CN column). Experimental details are described in the Materials and methods section.

sequences were obtained for the peptides by Edman degradation: I, Trp-Met-Asp-Phe; II, Trp-Met; III, Trp-Met. In peptides II and III, no peak was identified after the third cycle of sequencing. Peptides II and III were then acid-hydrolysed and the amino acid compositions determined. Both contained tryptophan, methionine, aspartic acid and phenylalanine at a molar ratio of 0.4:1.0:1.0:1.0 (the loss of tryptophan at this level was inevitable in the analytical conditions employed). On m.s. analysis, peaks I, II and III showed molecular ions of $(M+H)^+$ at m/z 597, 579 and 597 respectively. A similar observation was obtained on modification of another peptide, neurokinin A (His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH₂). When this peptide was modified by EDC without nucleophilic reagent, dissolved in water and analysed by reversed-phase h.p.l.c., a peak of R_t 34 min appeared after the unmodified peptide (R_t 30 min) as a major product (results not shown). On analysis of the modified peptides which had been dissolved in 100 mM Tris/HCl, pH 8.0, the peak at R_t 34 min disappeared and a new peak with R_t 29 min appeared before the unmodified peptide. During amino acid sequencing, both peptides (R_t 34 min and 29 min) were sequenced up to Thr-3 (as His-Lys-Thr). In this case also no peak was identified after the fourth cycle of sequencing. On m.s. analysis, the predominant peak was found to be due to the doubly charged ion (in electrospray ionization m.s., peptides and proteins with basic amino acids are known to give multiply charged ions [20]). In this

case, the unmodified peptide had a peak of $(M+2H)^{2+}$ at m/z 567 (meaning that the M_r of the peptide was 1132). Modified peptides of R_t 34 min and R_t 29 min had peaks of $(M+2H)^{2+}$ at m/z 558 and 567, from which M_r values of 1114 and 1132 respectively were determined. Amino acid composition and m.s. analysis indicate that the modified peptides were not cleaved, despite exhibiting only part of the amino acid sequences of the unmodified peptides (structures of the modified peptides are discussed below). Finally, all these results suggest that, in *Penicillium* 1,2- α -mannosidase, a small peak appearing on reversed-phase h.p.l.c. (peak A in Figure 4) was the result of modification of a carboxy group (Asp-4) in the adjacent peptide (peak B in figure 4).

dMM has an imino group itself and theoretically it can act as a nucleophile during modification. We modified CCK tetrapeptide by EDC in the presence or absence of dMM, and the reaction products were compared. No new peak was produced by adding dMM to the reaction (results not shown), meaning that, under the conditions employed, dMM did not act as a nucleophile. dMM was proposed merely to block the carboxy group during modification in its presence, and not to be incorporated into the enzyme.

DISCUSSION

At present, we cannot define the number of carboxy groups crucial for α -mannosidase activity, but several results have made us think that several, at least two, might be involved. When chemical modification was performed in the presence of dMM, a competitive inhibitor of the enzyme, inactivation was partially prevented; it is not clear why the activity was not completely protected. One possible explanation is that dMM does not mask the entire catalytic site during the modification. This would infer that the enzyme may have two catalytically involved amino acids, one of which can be masked by dMM. This might explain why the inhibition of the *Penicillium* enzyme by dMM was not linear above 1 mM, and approx. 10 % of the activity was observed even in the presence of higher concentrations (up to 5 mM) of the drug [12]. Another idea is that dMM is not a very potent inhibitor and that EDC has many chances to react with the catalytic carboxy group. Indeed, the K_i value of 105 μ M for the *Penicillium* enzyme is higher than those for mammalian enzymes [22]. In the case of glucoamylase from *Aspergillus awamori*, the enzyme was known to be inhibited by 1-deoxynojirimycin with a K_i of 96 μ M [24], a much higher concentration than for mammalian enzymes [25]. However, Harris et al. [26] demonstrated in crystallographic work that two molecules of 1-deoxynojirimycin bind to the catalytic site, one of which interacts with Glu-400, a catalytic base of that enzyme. In the *Penicillium* enzyme, we cannot yet define the mechanism of protection, but it seems experimentally possible to determine the catalytically important carboxy group using dMM as a protecting reagent.

In the absence of GEE, most of the enzyme activity was lost after reaction with EDC alone, whereas approx. 20 % more inactivation occurred in the presence of GEE. dMM showed a similar extent of protection under both conditions. On the other hand, incorporation of EDAN was not affected by the presence of dMM during the modification. These results suggest that the enzyme might have at least two catalytically important carboxy groups, one that can be masked by dMM and needs no nucleophilic reagent in the modification, and one that might not be masked by dMM and incorporates a nucleophilic reagent. A similar observation was reported by Arikawa and Fukui [4] in the modification of rabbit muscle phosphorylase *b*. In that case, the enzyme had three carboxy groups that could react with 1-

Scheme 1

cyclohexyl-3-[2-morpholinyl-(4-ethyl)carbodi-imide, but only one incorporated *N*-(2,4-dinitrophenyl)ethylenediamine as nucleophile. They report that only one of the three carboxy groups was essential for enzyme activity.

On reversed-phase h.p.l.c. of modified enzymes after lysylendopeptidic digestion, two peaks (A and B in Figure 4) showed opposite responses to the presence of dMM during the modification. They had a common amino acid sequence, suggesting that peptide A might increase and peptide B might decrease by the modification of a common carboxy group. Studies involving modification of short peptides, CCK tetrapeptide and neurokinin A, have given support to this idea. Peptides modified by EDC without nucleophilic reagent could be separated from unmodified peptide by reversed-phase h.p.l.c. Mild alkaline treatment caused a change in the chromatographic pattern, the modified peptide appearing before the unmodified peptide. Once a carboxy group was modified, Edman degradation of the peptide did not proceed beyond the modified amino acid residue. In modification of a carboxy group by EDC without a nucleophile, *O*-acylisourea is known to produce a stable *N*-acylisourea structure [1]. On the other hand, *O*-acylisourea can cyclize to form another intermediate, an imide structure, which was proposed by Franzblau *et al.* [27]. In the modification of RNAase A, Riehm and Scheraga [3] suggested that imides might be produced by modifying the enzyme with water-soluble carbodi-imide (at low pH) in the absence of nucleophiles. Our results show a good agreement with this idea. After modification at an acidic pH, the *M_r* of the peptides decreased by 18, indicating that a water molecule had been withdrawn. Amino acid sequencing of the peptide was interrupted, but all the amino acids were recovered on acid hydrolysis. These suggested the formation of an imide (form II in Scheme 1). After mild alkaline treatment, the *M_r* of the peptide increased by 18, indicating incorporation of a water molecule. Normal amino acids were released by acid-hydrolysis, but in this case also, Edman degradation was stopped before the modified residue. This suggested that a free α -carboxy group (form III) was formed instead of the free β -aspartyl carboxy group. We presume that the imide is changed to an α -carboxy structure by treatment with trimethylamine during Edman degradation, and thus amino acid sequencing is interrupted at this residue.

In *Penicillium* 1,2- α -mannosidase, we assume that Asp-4 in peptide B (in Figure 4) was protected from modification in the presence of dMM, and that the residue plays a part in interaction with the substrate. The role of the residue in enzyme activity will be examined in more detail in further experiments using protein engineering or other types of modification such as affinity labelling.

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