Degradation of glucagon in isolated liver endosomes

ATP-dependence and partial characterization of degradation products

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Endosomes have recently been identified as one major site of glucagon degradation in intact rat liver. In this study, a cell-free system has been used to assess the role of ATP-dependent acidification in endosomal glucagon degradation and identify the glucagon products generated. Percoll gradient fractionation of Golgi-endosomal fractions prepared 10–30 min after injection of \([^{125}\text{I}]\)iodoglucagon showed a time-dependent shift of the radioactivity towards high densities. Regardless of time, the radioactivity was less precipitable by trichloroacetic acid (Cl\(_3\)Ac) at high densities than at low densities. Chloroquine treatment slightly increased the density shift of the radioactivity and decreased its Cl\(_3\)Ac-precipitability throughout the gradient. Incubation of endosomal fractions containing \([^{125}\text{I}]\)iodoglucagon in 0.15 M-KCl at 30 °C resulted in a time- and pH-dependent generation of Cl\(_3\)Ac-soluble radioactivity, with a maximum at pH 4 (t\(_1\), 7 min). At pH 5, 1,10-phenanthroline, bacitracin and p-chloromercuribenzoic acid partially inhibited \([^{125}\text{I}]\)iodoglucagon degradation. At pH 6–7, ATP stimulated \([^{125}\text{I}]\)iodoglucagon degradation by 5–10-fold and caused endosomal acidification as judged from Acridine Orange uptake. The effects of ATP were inhibited by chloroquine, monensin, N-ethylmaleimide and dansylcadaverine. Poly(ethylene glycol) (PEG) precipitation of the radioactivity associated with endosomes showed that lowering the pH below 5.5 caused dissociation of the glucagon–receptor complex, and that, regardless of incubation conditions, all degraded \([^{125}\text{I}]\)iodoglucagon diffused extraluminally. On h.p.l.c., at least three products less hydrophobic than \([^{125}\text{I}]\)iodoglucagon were identified in incubation mixtures along with moniodotyrosine. Radiosequence analysis of the products revealed one major cleavage located C-terminally to Tyr-13 and two minor cleavages affecting Thr-5–Phe-6 and Phe-6–Thr-7 bonds. It is concluded that glucagon degradation in liver endosomes is functionally linked to ATP-dependent endosomal acidification and involves several cleavages in the glucagon sequence.

INTRODUCTION

Studies with isolated hepatocytes [1–5] and intact liver in vivo [6] have shown that, after binding to its receptor at the cell surface, glucagon is rapidly degraded by a proteolytic process. Some degradation occurs while the glucagon–receptor complex is still at the cell surface, presumably as a result of the action of plasma membrane-associated proteinases [7–11]; the remaining degradation requires endocytosis of the complex and occurs intracellularly [1–3,6]. There is indirect evidence that, although initially suggested to occur in lysosomes [1,2], degradation of internalized glucagon occurs mainly, or at least is initiated, in endosomes [6]. First, little association of \([^{125}\text{I}]\)iodoglucagon injected in vivo with hepatic lysosomes is demonstrable in cell fractionation studies [6]. Second, degradation products of \([^{125}\text{I}]\)iodoglucagon are readily detectable in hepatic endosomal fractions containing internalized ligand, provided that these fractions are isolated after chloroquine treatment in vivo [6].

In the present study, the involvement of endosomes in the degradation of \([^{125}\text{I}]\)iodoglucagon taken up by rat liver in vivo has been evaluated using a cell-free system similar to that recently used with insulin [12,13]. The sensitivity of \([^{125}\text{I}]\)iodoglucagon degradation to ATP has been examined and correlated with ATP-induced endosomal acidification. In addition, \([^{125}\text{I}]\)iodoglucagon-degradation products have been isolated by h.p.l.c., and cleavage sites affecting the glucagon sequence N-terminally to Tyr-13 have been identified by radiosequence analysis.

MATERIALS AND METHODS

Chemicals

Percoll was from Pharmacia. Methylamine, dansylcadaverine, p-chloromercuribenzoic acid, iodoacetamide and benzamidine were from Sigma. The sources of other chemicals have been specified previously [6].

Preparation of \([^{125}\text{I}]\)iodoglucagon

\([^{125}\text{I}]\)Iodo-Tyr-10]glucagon and \([^{125}\text{I}]\)iodo-Tyr-13]glucagon, or an equimolar mixture of these two isomers, were prepared using lactoperoxidase and purified by h.p.l.c. as described previously [6].

Animals and injections

Male Sprague–Dawley rats weighing 180–200 g were obtained from Charles River France and were fasted for 18 h before being killed. \([^{125}\text{I}]\)iodoglucagon (15 × 10^{-6}–50 × 10^{-6} c.p.m.) was diluted into 0.5 ml of 0.15 M-NaCl containing 0.1 % (w/v) BSA and injected over 15 s in the penis vein under light diethyl ether anaesthesia. Except for radiosequence studies, a mixture of isomers labelled at positions 10 and 13 was used. Rats were killed from 10 to 30 min after injection.

In studies with chloroquine, this drug was dissolved in 0.15 M-NaCl and given as two intraperitoneal injections (2.5 mg/100 g body weight each) at 75 and 15 min before injection of \([^{125}\text{I}]\)iodoglucagon as described previously [6].

Preparation of a Golgi–endosomal fraction containing internalized \([^{125}\text{I}]\)iodoglucagon

A 'total' Golgi–endosomal fraction (density 1.03–1.14 g/cm^3) was isolated from liver homogenates in 0.25 M-sucrose according to a modification [14] of the method of Ehrenreich et al. [15]. In some experiments, N-ethylmaleimide (2 mM), bacitracin (1 mg/ml) and 1,10-phenanthroline (5 mM) were included in the homogenization medium to minimize the degradation of \([^{125}\text{I}]\)iodoglucagon which occurs during fractionation. 1,10-Phenanthroline, an inhibitor of metalloproteinases, was used because of
its ability to inhibit insulin degradation in isolated liver endosomes [12,13]. After isolation, the Golgi–endosomal fraction was either subfractionated by centrifugation in an iso-osmotic Percoll density gradient [14], or immediately used in cell-free degradation and dissociation studies.

**Cell-free assay for degradation of [[125]I]iodoglucagon in endosomes**

A Golgi–endosomal fraction isolated 20 min after injection of [[125]I]iodoglucagon in the absence of proteinase inhibitors was suspended (0.1–2 mg of protein/ml) in 0.15 M-KCl containing 5 mM-MgCl2 or -MgSO4, 25 mM-citrate/phosphate, pH 3–7.5, and when indicated 1 mM-ATP, 0.1 % Brij-35 and/or specific drugs. Samples were incubated at 30 °C with constant shaking for various lengths of time, after which the integrity of [[125]I]iodoglucagon was assessed by precipitation with 5 % (w/v) Cl3Ac in the presence of 1 mg of BSA or by h.p.l.c. For h.p.l.c. studies, the Golgi–endosomal fraction was acidified with acetic acid (20 %) and freeze-dried. The dry residue was resuspended in water (1 ml) containing 0.1 % trifluoroacetic acid (F3Ac) and 3 % acetic acid and centrifuged for 1 h at 20000 g; at least 95 % of the radioactivity remained in the supernatant. The latter was filtered on a 0.45 μm minifilter (Millipore) and then chromatographed on a micro Bondapak C18 column using as eluent a mixture of 0.1 % F3Ac in water (solvent A) and 0.1 % F3Ac in acetonitrile (solvent B), pumped at a flow rate of 2 ml/min. Elution was carried out with two sequential linear gradients: first, a gradient of 0–10 % solvent B (10 min); and then a gradient of 10–50 % solvent B (60 min). The eluate was monitored on line for radioactivity with a γ detector. The major labelled components in the eluate were collected, freeze-dried and submitted to radiosequence analysis by automated Edman degradation as described previously [6].

**Cell-free assay for dissociation of [[125]I]iodoglucagon from endosomes**

Dissociation of [[125]I]iodoglucagon from endosomes was assessed by poly(ethylene glycol) (PEG) precipitation in the absence or presence of the detergent Brij-35 [16], basically as described in similar studies with insulin [12,13]. In intact endosomes, both receptor-bound and free intraluminal glucagon are PEG-precipitable, whereas in Brij-35-permeabilized endosomes, only receptor-bound glucagon is PEG-precipitable. After incubation of endosomal fractions containing [[125]I]iodoglucagon for 5 min in buffered 0.15 M-KCl at 30 °C as described above, Brij-35 (0.1 %, final) or KCl (0.15 M) was added. After 10 min at 4 °C with constant shaking, a PEG solution (12.5 %, w/v) in 50 mM-Tris/HCl buffer containing bovine γ-globulin (1 mg per sample) was added, and the resulting precipitate was pelleted and counted for radioactivity.

**Protein and enzyme assays**

Protein was measured according to Lowry et al. [17] using BSA as standard. ATP-dependent endosomal acidification was measured using Acidride Orange as described previously [12]. 5'-Nucleotidase [18], galactosyltransferase [19] and acid phosphatase [20] were measured according to previously described methods.

**RESULTS**

**Subfractionation of hepatic Golgi–endosomal fractions containing internalized [[125]I]iodoglucagon**

It has previously been shown that, after injection of [[125]I]iodoglucagon into rats, the radioactivity taken up by the liver is concentrated in Golgi–endosomal fractions with a maximum at 20 min [6]. It was also found that the radioactivity present in these fractions is associated with structures physically separable from Golgi elements and lysosomes, presumably endosomes [6]. To evaluate the contribution of individual endosomal components to [[125]I]iodoglucagon uptake and degradation in vivo, a total Golgi–endosomal fraction, prepared various times after [[125]I]iodoglucagon injection, was subfractionated on Percoll density gradients (Fig. 1, open histograms). At 10 min after injection, most of the radioactivity was recovered in the upper half, low-density region of the gradient, coinciding in part with 5'-nucleotidase (a plasma-membrane marker) and galactosyltransferase (a Golgi marker). Later, the radioactivity was more broadly distributed throughout the gradient, overlapping to a large extent with acid phosphatase (a lysosomal marker). This shift was maximal at 20 min and was still detectable at

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**Fig. 1. Distribution of enzymes and radioactivity in Golgi–endosomal Percoll gradient subfractions from control (□) and chloroquine-treated (■) rats after [[125]I]iodoglucagon injection**

A Golgi–endosomal fraction was prepared from rats killed at the indicated times after injection of [[125]I]iodoglucagon (about 5 × 107–10 × 108 c.p.m.) and subfractionated by Percoll density gradient as described in the Materials and methods section. Fourteen fractions (0.5 ml each) were collected and analysed for enzyme activity or radioactivity, with results expressed as % of the total recovered from the gradient. The profiles shown are representative of at least two experiments performed in separate liver fractionations. Densities of gradient subfractions were as reported previously [14].
Fig. 2. Cl$_3$Ac solubility of the radioactivity associated with Golgi-endosomal Percoll gradient subfractions from control (a) and chloroquine-treated (b) rats after $[^125]$iodoglucagon injection

Golgi-endosomal Percoll gradient subfractions were prepared from rats killed 10 min (a), 20 min (b) or 30 min (c) after injection of $[^125]$iodoglucagon as described in the legend to Fig. 1. In each subfraction, the percentage of radioactivity remaining soluble in 5% Cl$_3$Ac was determined.

Fig. 3. Effect of ATP on the pH-dependence of endosomal $[^125]$iodoglucagon degradation in the presence of various drugs

A Golgi-endosomal fraction was isolated 20 min after injection of $[^125]$iodoglucagon (about $30 	imes 10^6$ c.p.m.). Samples were suspended in 0.15 M-KCl (a and c–h) or 0.25 M-sucrose (b), containing 25 mM-citrate/phosphate at the indicated pH. In some cases, one of the following drugs was included at the indicated concentration: (c) chloroquine, 20 mM; (d) dansylcadaverine, 5 mM; (e) N-ethylmaleimide 1 mM; (f) monensin, 10 µM; (g) vanadate, 0.1 mM; (h) ouabain, 1 mM. These suspensions were incubated for 5 min at 30°C in the absence (□) or presence (■) of 1 mM-ATP and 5 mM-Mg$^{2+}$ ions either as chloride (KCl medium) or sulphate (sucrose medium). The percentage of $[^125]$iodoglucagon degraded during incubation was estimated by Cl$_3$Ac precipitation, with results corrected for the amount of Cl$_3$Ac-soluble radioactivity present initially (about 10%). The results shown are the means of three to five determinations performed in separate liver fractionations.

30 min. Analysis of the radioactivity associated with Percoll gradient subfractions showed a slight time-dependent increase in Cl$_3$Ac-solubility, which was maximal 20 min after the injection (Fig. 2). Regardless of time, more radioactivity was Cl$_3$Ac-soluble in high-density subfractions (15–25%) than in low-density subfractions (5–15%).

In previous studies, chloroquine treatment in vivo has been shown to cause a slight increase in the late recovery of injected $[^125]$iodoglucagon in Golgi-endosomal fractions and a paradoxical increase in the Cl$_3$Ac solubility of the radioactivity [6]. Analysis of the distribution of the radioactivity on Percoll gradients showed that, at 20 min, chloroquine augmented the shift of the radioactivity towards high densities (Fig. 1, solid histograms). Under similar conditions, the pattern of distribution of marker enzymes was unaffected. Additionally, chloroquine treatment increased the Cl$_3$Ac solubility of the radioactivity throughout the gradient, with a maximal effect (2–3-fold over control rats) at 10 min (Fig. 2).

Cell-free degradation of $[^125]$iodoglucagon in intact endosomes

Golgi-endosomal fractions isolated 20 min after $[^125]$iodoglucagon injection were incubated at 30°C under various time and pH conditions, after which the percentage of radioactivity converted into Cl$_3$Ac-soluble products was determined. As shown
in Fig. 3, the generation of Cl4Ac-soluble radioactivity from [125I]iodoglucagon occurred mainly in the pH interval 3–5, with a sharp maximum at pH 4 (initial rate of degradation, 7.23 ± 0.19 % of the total per min; half-life, 6.9 min). There was little degradation of [125I]iodoglucagon at pH above 6 and 7 (rates, about 13 and 8 % of the total per h respectively; Fig. 5a). Regardless of pH, less degradation occurred in 0.25 M-sucrose than in 0.15 M-KCl (Fig. 3).

Table 1. Effect of weak bases and proteinase inhibitors on the degradation of [125I]iodoglucagon in cell-free endosomes

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
<th>[125I]iodoglucagon degraded (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Weak bases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloroquine</td>
<td>10 mm</td>
<td>72.4 ± 4.10</td>
</tr>
<tr>
<td>Dansylcadaverine</td>
<td>6 mm</td>
<td>53.3 ± 3.20</td>
</tr>
<tr>
<td>Methylamine</td>
<td>10 mm</td>
<td>99.2 ± 1.24</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>50 mm</td>
<td>112.1 ± 2.10</td>
</tr>
<tr>
<td>Proteinase inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
<td>1 mm</td>
<td>66.5 ± 3.95</td>
</tr>
<tr>
<td></td>
<td>5 mm</td>
<td>25.8 ± 1.47</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>3 mg/ml</td>
<td>32.8 ± 0.10</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate</td>
<td>1 mm</td>
<td>66.7 ± 2.72</td>
</tr>
<tr>
<td>Phenylmethanesulphonyl fluoride</td>
<td>1 mm</td>
<td>97.2 ± 2.90</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>3 mm</td>
<td>95.0 ± 3.98</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>3 mm</td>
<td>96.8 ± 2.82</td>
</tr>
</tbody>
</table>

Table 2. Effect of various drugs on ATP-dependent acidification of the Golgi–endosomal fraction

<table>
<thead>
<tr>
<th>Addition</th>
<th>Relative decrease in fluorescence intensity (% of initial)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>-ATP    +ATP</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3.0±1.0  6.2±0.1</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>5.2±0.6  5.3±0.2</td>
</tr>
<tr>
<td>Dansylcadaverine</td>
<td>5.8±1.2  4.1±3.0</td>
</tr>
<tr>
<td>Monensin</td>
<td>3.2±0.2  4.8±0.2</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>3.8±0.25 4.8±0.3</td>
</tr>
<tr>
<td>Vanadate</td>
<td>4.6±0.1  5.0±0.4</td>
</tr>
<tr>
<td>Ouabain</td>
<td>3.6±0.3  6.9±0.1</td>
</tr>
<tr>
<td></td>
<td>3.9±0.1  6.5±0.5</td>
</tr>
</tbody>
</table>

At pH 5, degradation of [125I]iodoglucagon within isolated liver endosomes was partially inhibited by chloroquine and dansyl-cadaverine, two acidotropic agents, and by 1,10-phenanthroline, bacitracin and p-chloromercuribenzoic acid, three proteinase inhibitors (Table 1). It was, however, unaffected by methylamine, NH4Cl, iodoacetamide, phenylmethanesulphonyl fluoride and benzamidine.

Addition of ATP (1 mM) to the cell-free system broadened the pH range at which degradation of [125I]iodoglucagon occurred, stimulating degradation by 5–10-fold at pH above 5.5 (Fig. 3 and Fig. 5b). However, neither the pH at which the degradation was maximal nor the rate of degradation at the optimum pH were significantly affected by ATP. The ability of ATP to stimulate [125I]iodoglucagon degradation at pH 5–7 did not occur if sucrose was substituted for KCl, suggesting a requirement for Cl- ions (Fig. 3). The effect of ATP was also suppressed by chloroquine and dansyl-cadaverine, monensin (a proton ionophore) and N-ethylmaleimide (an inhibitor of the vacuolar-type ATPase) (Fig. 3). In contrast, ATP was fully effective in the presence of vanadate and ouabain (two inhibitors of Na+/K+-ATPase) (Fig. 3). Concentrations of ATP of 1, 3 and 6 mM were equally effective in stimulating [125I]iodoglucagon degradation, and neither ADP nor adenosine 5'-[βy-imido]triphosphate, a non-hydrolysable analogue of ATP, could substitute for ATP (results not shown).

At pH 6, at which [125I]iodoglucagon degradation was maximally stimulated by ATP, this nucleotide caused endosomal acidification, as judged by the uptake of Acridine Orange by endosomes (Table 2). Furthermore, drugs that suppressed the effect of ATP on [125I]iodoglucagon degradation also inhibited ATP-dependent acidification, suggesting that ATP acted on degradation via endosomal acidification (Table 2).
Fig. 5. Effect of ATP on the time-dependence of dissociation and degradation of \(^{125}\text{I}\)iodoglucagon associated with endosomes

Samples of a Golgi-endosomal fraction were suspended in 0.15 M-KCl, 25 mM-citrate/phosphate at pH 6 ( ), or 7 ( ). At pH 5.5, the percentage of \(^{125}\text{I}\)iodoglucagon dissociated and degraded was determined by PEG and ClAc precipitation respectively. The results shown are the means of two determinations performed on separate liver fractionations.

Fig. 6. H.p.l.c. elution profiles of the radiolabelled products generated from \(^{125}\text{I}\)iodoglucagon in endosomes incubated for various times at pH 4

Samples of a Golgi-endosomal fraction isolated 20 min after \(^{125}\text{I}\)iodo-Tyr-13glucagon (30 x 10^6 c.p.m.) were incubated in 0.15 M-KCl containing 25 mM-citrate/phosphate, pH 4, and incubated for 5 (a), 10 (b) and 20 (c) min at 30 °C. The radioactivity was then acid-extracted and analysed by h.p.l.c. as described in the Materials and methods section. The major degradation products identified at 20 min, numbered sequentially 1, 2 and 3, were eluted at 50, 55 and 57 min respectively. Monoiodotyrosine (thin arrow) and intact \(^{125}\text{I}\)iodoglucagon (thick arrow) were eluted at 18 and 70 min respectively. The profiles shown are representative of at least six experiments performed in separate liver fractionations. Identical elution profiles were obtained when \(^{125}\text{I}\)iodo-Tyr-10glucagon was used as a ligand (not shown).

Relationships between \(^{125}\text{I}\)iodoglucagon dissociation and degradation

The effect of pH on the dissociation of \(^{125}\text{I}\)iodoglucagon from endosomes was examined using the PEG-purification procedure (Figs. 4c and 4d). In intact and freshly isolated endosomes, about 80% of the radioactivity was PEG-purifiable. After incubation for 5 min at 30 °C, a marked increase in PEG-soluble radioactivity was observed below pH 5.5, with a maximum at pH 4 (about 80%). Regardless of pH but especially at low pH, the amount of PEG-soluble radioactivity exceeded the amount of ClAc-soluble radioactivity (by about 15% at pH 5-7 and up to 30%, at pH 4). In the presence of 1,10-phenanthroline, which inhibited the increase in ClAc-soluble radioactivity, an increase in PEG-soluble radioactivity still occurred below pH 5.5, but to a lesser extent. These findings suggest that, regardless of pH, all degraded \(^{125}\text{I}\)iodoglucagon diffuses out of endosomes. However, some dissociation of intact \(^{125}\text{I}\)iodoglucagon also occurs at low pH, presumably as a result of an enhanced endosomal permeability.

To distinguish between receptor-bound and free intraluminal \(^{125}\text{I}\)iodoglucagon, the experiments described above were repeated with Brij-35-permeabilized endosomes (Figs. 4c and 4d). Although under these conditions slightly more radioactivity became PEG-soluble at pH values above 5.5, the relative increase in PEG-soluble radioactivity observed when the pH was lowered to 4-4.5 was nearly the same as in non-permeabilized endosomes.

However, a marked increase in PEG-soluble radioactivity was observed at pH 5, indicating that, at this pH, a substantial fraction of \(^{125}\text{I}\)iodoglucagon associated with endosomes was free in the endosomal lumen, presumably in an intact form. Furthermore, the increase in PEG-soluble radioactivity observed at lower pH was the same whether 1,10-phenanthroline was present or not, indicating that degradation is not required for acid-triggered dissociation of the glucagon-receptor complex.

At pH 6 and 7, ATP increased the rate of formation of PEG-soluble radioactivity almost identically with the rate of formation of ClAc-soluble radioactivity (Fig. 5). Thus degradation products of \(^{125}\text{I}\)iodoglucagon generated on ATP-induced endosomal acidification diffused quantitatively out of endosomes.

Characterization of \(^{125}\text{I}\)iodoglucagon-degradation products generated in cell-free endosomes

The degradation products generated from \(^{125}\text{I}\)iodo-Tyr-10glucagon and \(^{125}\text{I}\)iodo-Tyr-13glucagon in cell-free endosomes
Radiolabelled degradation products generated from mono\[^{[125I]}\]iodo-Tyr-10\]glucagon in cell-free endosomes at pH 4

Radiolabelled degradation products generated from mono\[^{[125I]}\]iodo-Tyr-10\]glucagon in cell-free endosomes at pH 4 and isolated by h.p.l.c. (see Fig. 6) were submitted to automated radiosequence analysis as described in the Materials and methods section. (a) and (c) show the radiosequence analysis of peptides 1 and 3 respectively, generated from \[^{[125I]}\]iodo-Tyr-10\]glucagon. (b) and (d) show radiosequence analysis of peptides 2 and 3 respectively, generated from \[^{[125I]}\]iodo-Tyr-13\]glucagon.

Fig. 7. Automated radiosequence analysis of radiolabelled degradation products generated from mono\[^{[125I]}\]iodoglucagons in cell-free endosomes at pH 4

Samples of a Golgi–endosomal fraction containing internalized \[^{[125I]}\]iodoglucagon were suspended in 0.15 \(\text{m-KCl}\) containing 25 \(\text{mm-citrate-phosphate}\) at the indicated pH, and incubated for 5 min at 30 °C in the absence (a) or presence (b) of 1 \(\text{mm-ATP}\) and 5 \(\text{mm-MgCl}_2\). After acid extraction and h.p.l.c. analysis (see the Materials and methods section), the radioactivity eluted as intact \[^{[125I]}\]iodoglucagon (□), \[^{121I}\]labelled intermediate products (▲) and monoidotyrosine (■) was measured. The results shown are means ± S.E.M. of three to five experiments performed on separate liver fractionations.

Fig. 8. Quantification of the radiolabelled products generated from \[^{[125I]}\]iodoglucagon in cell-free endosomes as a function of time

were isolated by h.p.l.c. Representative elution profiles obtained after incubation for 5, 10 and 20 min at pH 4 are shown in Fig. 6. In addition to monoiodotyrosine and intact \[^{[125I]}\]iodoglucagon, three major degradation products less hydrophobic than \[^{[125I]}\]iodoglucagon, numbered sequentially 1–3, were identified in the eluate. Product 3, the most hydrophobic (retention time, 57 min), was the only product detectable at 5 min and the main product generated at 10 and 20 min. Products 1 and 2 (retention times, 50 and 55 min respectively) were detectable at later times and were less abundant than product 3, especially the latter.

Products 1, 2 and 3 were collected and submitted to automated Edman degradation to determine the cycle at which the radioactivity was released (Fig. 7). With \[^{[125I]}\]iodo-Tyr-10\]glucagon (Figs. 7a and 7c), the radioactivity associated with products 1 and 3 was released during cycles 4 and 10 respectively; some radioactivity associated with product 1 was also released during cycles 1 and 3. With \[^{[125I]}\]iodo-Tyr-13\]glucagon (Figs. 7b and 7d), the radioactivity associated with products 2 and 3 was released mainly during cycles 8 and 13 respectively. These results identify residues Thr-7, Phe-6 and His-1 as the \(N\)-terminal residues of products 1, 2 and 3 respectively, and indicate that the cleavage which generates product 3 is located C-terminally to residue Tyr-
DISCUSSION

Using subcellular fractionation, we have recently shown that glucagon taken up by rat liver in vivo accumulates in part in low-density endosomal components recovered from the microsomal fraction [6]. We have also found that, although most of the \( {^{125}I} \)iodoglucagon extracted from freshly prepared endosomal fractions retains integrity, \( {^{125}I} \)iodoglucagon-degradation products are readily detectable after chloroquine treatment of rats in vivo. Using a cell-free system, we show here that the degradation of \( {^{125}I} \)iodoglucagon in intact liver endosomes is functionally linked to ATP-dependent endosomal acidification and involves at least three cleavages in the glucagon sequence. These observations extend recent findings on the degradation of insulin in liver endosomes [12,13] and indicate that glucagon is an equally good substrate for (the) endosomal proteinase(s).

It has previously been shown that liver endosomes containing internalized insulin [21] and prolactin [22] can be resolved into components of low and high density by Percoll density-gradient centrifugation. Using a similar approach, we show here that, like these ligands, injected \( {^{125}I} \)iodoglucagon accumulates earlier in low-density than in high-density endosomal components. Although co-sedimenting with galactosyltransferase, the ‘early’ low-density components appear to be distinct from Golgi elements as judged from our previous study using the diamino-benzidine density-shift method [6]. The present study also shows that, regardless of post-injection time, more radioactivity is present in the Cl\(_2\)Ac-soluble form in high-density endosomal components than in low-density components. This observation suggests that either high-density endosomes degrade glucagon to a greater extent than low-density endosomes or, because of their smaller size, degradation products generated in low-density endosomes diffuse extraluminally.

We have recently shown that, in glucagon-injected rats, chloroquine treatment causes a moderate but significant accumulation of internalized \( {^{125}I} \)iodoglucagon in Golgi-endosomal fractions [6]. Such treatment also caused a paradoxical increase in the fraction of radioactivity recovered as intermediate degradation products, suggesting that a low pH was required for conversion of these products into monoiiodotyrosine. We show here that, although increasing the association of internalized \( {^{125}I} \)iodoglucagon to high-density endosomes, chloroquine treatment leads to a similar increase in Cl\(_2\)Ac-soluble radioactivity in both endosome populations. These observations are consistent with the finding that, despite one report showing that only high-density endosomes concentrate chloroquine in vitro [22], both endosome populations undergo a comparable ATP-dependent acidification in vitro [12].

Various ligands internalized by receptor-mediated endocytosis, such as insulin in liver [12,13] and mannosylated serum albumin in macrophages [23,24], have been shown to undergo degradation in cell-free endosomes maintained at a low pH. Furthermore, addition of ATP to endosomes, by acidifying their internal content, was shown to augment ligand degradation at neutral pH. The present study extends these observations to glucagon and shows that, in the presence of ATP, a significant increase in glucagon degradation occurs at pH 7, a pH value comparable with that achieved in the cytoplasm. However, degradation of \( {^{125}I} \)iodoglucagon in isolated liver endosomes differed from that of \( {^{125}I} \)iodoinsulin in at least two respects: first, a lower pH for maximal degradation (4 instead of 5); and second, a lesser effect of ATP on degradation at neutral pH. As discussed below, this presumably reflects the fact that a lower pH is required for dissociation of the glucagon-receptor complex, and that the decrease in internal pH induced by ATP does not allow full dissociation of the complex.

Evidence has been presented that the increased degradation of insulin [12,13] and mannosylated serum albumin [23,24] which occurs in endosomes at a low pH and/or in the presence of ATP is accompanied, and presumably mediated, by an increased dissociation of these ligands from their receptors. Using a PEG-precipitation procedure comparable with that used with these ligands, we show here that lowering the pH also causes dissociation of the glucagon-receptor complex, with a maximum at pH 4. Furthermore, there was a narrow pH interval (about pH 5) at which complex dissociation, but not ligand degradation, was increased, and the pH-dependence of dissociation was the same whether \( {^{125}I} \)iodoglucagon degradation was inhibited or not. Taken together, these observations suggest that the acid-triggered dissociation of the glucagon-receptor complex is required for \( {^{125}I} \)iodoglucagon degradation. Free intraluminal \( {^{125}I} \)iodoglucagon serving as a substrate for (the) endosomal proteinase(s). Once formed, virtually all \( {^{125}I} \)iodoglucagon-degradation products appear to diffuse out of endosomes.

The sensitivity of the endosomal glucagon-degrading process to selected proteinase inhibitors and weak bases is comparable with that observed in previous studies with insulin [12,13,16,25]. Thus, like insulin degradation, glucagon degradation was inhibited by 1,10-phenanthroline, \( p \)-chloromercuribenzoic acid and bacitracin, but was unaffected by phenylmethanesulphonyl fluoride and benzamidine. These results suggest that the degradation of insulin and glucagon within liver endosomes is catalysed by (a) similar enzyme(s), and that at least one enzyme involved is a thiol proteinase requiring a metal ion for optimum activity.

Characterization of the labelled products generated from \( {^{125}I} \)iodo-Tyr-10glucagon and \( {^{125}I} \)iodo-Tyr-13glucagon in cell-free endosomes allowed the identification of three cleavage sites: one major site, located C-terminally to Tyr-13; and two minor sites, located at the Thr-5–Phe-6 and Phe-6–Thr-7 bonds. In our previous study on chloroquine-treated rats, cleavages at bonds Thr-5–Phe-6 and Phe-6–Thr-7 were also identified in freshly extracted endosomal fractions. However, under these
conditions no cleavage site was detectable C-terminally to Tyr-13
and an additional cleavage was identified at bond Ser-2-Gln-3.
These discrepancies may result from the fact that the internal
endosomal pH achieved in vivo after chloroquine treatment is
higher than that achieved in vitro on exposure to an acidic
medium. In addition, some degradation products formed in vitro
may rapidly diffuse out of endosomes and thus escape subsequent
detection.

At a first glance, the endosomal glucagon-degrading activity
described here differs in several respects from other glucagon-
degrading activities identified in isolated hepatocytes and liver
plasma membranes. Thus it differs from: the activity identified
by Hagopian and Tager [4,5] in rat and canine hepatocytes,
which cleaves glucagon at the Gln-3-Gly-4 bonds; the receptor-
linked proteinase identified by Sheetz and Tager [9,10] in canine
plasma membranes, which cleaves glucagon at several sites C-
terminally to Tyr-13 and is inhibited by phenylmethylsulphonyl
fluoride; and the thiol endopeptidase activity identified by Blache
et al. [11] in rat plasma membranes, which generates the 19–29
fragment and is unaffected by bacitracin. The endosomal glu-
cagon-degrading activity appears to differ also from cytosolic
insulin proteinase, which was recently shown to cleave selectively
the Arg-17–Arg-18 bond [26]. However, identification of the
cleavage sites located beyond Tyr-13 will be required to evaluate
fully the involvement of the enzymes described above in endo-
somal glucagon degradation. At this stage, it appears likely that
more than one enzyme is involved in this process.

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