A lysate of purified insulin secretory granules, which contains two types of proinsulin processing activity (type I, Arg-Arg-directed and type II, Lys-Arg-directed (Davidson, H. W., Rhodes, C. J., and Hutton, J. C. (1988) Nature 333, 83-96), was found to process proalbumin by specific proteolytic cleavage of the COOH-terminal side of the Arg$^2$-Arg$^1$ sequence. The subclass distribution of proalbumin processing activity in insulinoma tissue paralleled that for proinsulin conversion and occurred principally in a secretory granule fraction. Cleavage appeared to result from the Arg-Arg-directed type 1 proinsulin processing endopeptidase. It was Ca$^{2+}$-dependent ($K_{0.5}$ activation = 1.0-1.5 mM Ca$^{2+}$), unaffected by group-specific inhibitors of serine, cysteinyl, or asparaginyl proteinases, and had an acidic pH optimum (pH 5.5). Active-site inhibitor studies showed this activity had a preference for dibasic over monobasic amino acid sequences and indicated that the sequence of the dibasic site was an important determinant of the susceptibility of the substrate to cleavage. The activity did not process the proalbumin Christchurch mutant (Arg$^1$-Arg$^2$ to Arg$^1$-Arg$^1$-Gln$^3$). It was inhibited by the variant $\alpha_1$-antitrypsin Pittsburgh (Met$^{256}$ to Arg$^{256}$; $K_{0.5}$ = 100 nM) but not by other related proteins normally co-secreted with albumin from hepatocytes, namely $\alpha_1$-antitrypsin M, $\alpha_2$-macroglobulin, or antithrombin III. The insulin secretory granule proalbumin processing activity was indistinguishable from a proalbumin endopeptidase reported in rat liver membranes and similar to the yeast KEX-2 protease. These findings suggest that a highly conserved set of proprotein endopeptidases exists, which are specific for a dibasic sequence but broadly specific for proprotein substrates. Such enzymic activities appear to be active within both the constitutive and regulated pathways of secretion. Intraorganelar Ca$^{2+}$ and pH appear to play a key role in regulating their activities.

Many secreted proteins are synthesized as larger inactive precursors and processed post-translationally by limited proteolysis at sites marked by paired basic amino acids (1-3). This applies to both constitutively secreted molecules, e.g. albumin (4, 5), and proteins stored intracellularly in vesicles which undergo exocytosis in response to stimulus of the cell, e.g. insulin (6, 7).

Albumin is produced from its precursor proalbumin in hepatocytes by proteolytic removal of the NH$_2$-terminal hexapeptide Arg-Gly-Val-Phe-Arg-Arg (8-10). This process occurs intracellularly in the trans-Golgi network/secretory vesicle compartments (4, 5) and is catalyzed by a Ca$^{2+}$-dependent endopeptidase activity with an acidic pH optimum (11).

The major site of proteolytic conversion of proalbumin in the pancreatic $\beta$-cell is the insulin secretory granule (12-15). Cleavage occurs on the carboxylic side of two dibasic sequences in the proalbumin molecule at Arg$^3$-Arg$^3$ and at Lys$^4$-Arg$^6$ (16). It is catalyzed by Ca$^{2+}$-dependent endoproteolytic activities of acidic pH optima (17) followed by the action of carboxypeptidase H (18, 19) that trims off the COOH-terminal basic amino acid residues generated by the endopeptidase clips. Studies on lysates of purified insulinoma secretory granules have revealed that two endopeptidase activities are involved (20). One (type I) cleaves exclusively at the Arg-Arg site, the proalbumin B/C-chain junction. The other (type II) preferentially cleaves at the Lys-Arg site, the proalbumin A/ C-chain junction, though it will also recognize the Arg-Arg site to a lesser extent (10-20%).

It is not clear whether the proteolytic processing of proproteins at dibasic sequences, exemplified by proalbumin and proinsulin, occurs as a consequence of the activity of a limited number of broadly specific widely distributed enzymes or a large group of tissue- and substrate-specific enzymes. In addition, it is not clear whether proteolytic conversion activity directed at constitutively secreted proteins differs from that directed at proteins which are segregated at the level of the trans-Golgi network for storage into secretory granules. We have addressed these questions through the investigation of the conversion of proalbumin (a constitutively secreted protein (7)) by intracellular elements of the pancreatic $\beta$-cell in which the regulated pathway predominates (6).

**EXPERIMENTAL PROCEDURES**

**RESULTS**

The conversion of proalbumin to albumin by insulin secretory granule extracts is illustrated in Fig. 1. The loss of the positively charged propeptide causes an increase in anodal mobility of the protein on agarose gel electrophoresis and results in the ability to bind $^{63}$Ni. Binding of $^{63}$Ni requires a free $\alpha$-amino group on residue 1 and a histidine in position 8, viz. Asp-Ala-His, and therefore provides confirmation that

* Portions of this paper (including "Experimental Procedures" and Fig. M1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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Christopher J. Rhodes, Stephen O. Brennan, and John C. Hutton*  
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† Juvenile Diabetes Foundation International Research Fellow.

‡ To whom correspondence should be addressed.

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Proalbumin processing by insulin secretory granule lysates. Conversion of proalbumin (5 µg) was determined after 3 h at 30 °C by Coomassie Blue staining (lower panel) and 56Ni autoradiography (upper panel) of a 1% (w/v) agarose gel as described (see "Experimental Procedures"). The lanes are as follows: lane 1, proalbumin only; lane 2, proalbumin + insulin secretory granules + EDTA; lane 3, proalbumin + insulin secretory granules; lane 4, proalbumin + insulin secretory granules + 0.5 µg of α1-antitrypsin M (normal); lane 5, proalbumin + insulin secretory granules + 0.5 µg of α1-antitrypsin Pittsburgh; lane 6, proalbumin + insulin secretory granules + 0.5 µg of antithrombin III; lane 7, proalbumin + insulin secretory granules + 0.5 µg of α1-macroglobulin; lane 8, proalbumin Christchurch + insulin secretory granules; lane 9, proalbumin Christchurch only; lane 10, insulin secretory granules only; lane 11, proalbumin + insulin secretory granules (1-h incubation at 30 °C); lane 12, plasma from the heterozygous carrier of proalbumin Christchurch.

Proalbumin processing activity in different subcellular fractions of pancreatic β-cell tissue paralleled that for proinsulin processing activity (Fig. 2). The majority of proalbumin (and proinsulin) processing was found in the insulin secretory granule fraction (fraction Ib), which consisted of highly purified insulin secretory granules and contaminating mitochondria. Little proalbumin (and proinsulin (17)) processing was found in the other β-cell subcellular fractions (including the lysosome-rich fractions (Ia)). The rate of proalbumin conversion activity by each subcellular fraction was assayed at 30 °C for 3 h as described and is shown in panel A as a mean ± S.E. (n = 4). Similarly, 125I-proalbumin processing activity in each of these fractions (50 µg of protein) was assayed at 30 °C for 3 h as described and is shown in panel B as a mean ± S.E. (n = 4). Panel C shows the mean insulin content ± S.E. (n = 3) of the insulinoma subcellular fractions.

Proalbumin conversion by a proinsulin processing endopeptidase. Proalbumin processing activity in subcellular fractions of rat insulinoma tissue. Subcellular fractionations were prepared by Percoll density gradient centrifugation as previously described (see "Experimental Procedures"). The fractions contained the following organelles (as determined by marker enzyme analysis): Ia, lysosomes; Ib, insulin secretory granules; Ic, insulin secretory granules/mitochondria; II, mitochondria/endoplasmic reticulum; III, endoplasmic reticulum/Golgi apparatus; IV, plasma membrane/endoplasmic reticulum; V, cytosol. 125I-Proalbumin to 125I-albumin processing activity by each subcellular fraction (50 µg protein) was assayed at 30 °C over 2 h as described and is shown in panel A as a mean ± S.E. (n = 4). Similarly, 125I-proinsulin processing activity in each of these fractions (50 µg of protein) was assayed at 30 °C for 3 h as described and is shown in panel B as a mean ± S.E. (n = 4). Panel C shows the mean insulin content ± S.E. (n = 3) of the insulin subcellular fractions.

Proinsulin processing type I (Arg-Arg-directed) and type II (Lys-Arg-directed) endopeptidase activities can be separated by anion-exchange chromatography (20) (Fig. 3). The processing of proalbumin to albumin was associated principally with the Arg-Arg-directed type I activity and with a minor component co-eluting with the type II endopeptidase activity. The relative ratio of proalbumin processing by the type I and type II endopeptidase was also 9:1, which coincided with the relative rates for the Arg-Arg-directed cleavage of proalbumin by the same fractions.

The proalbumin processing activity of insulin secretory granules was Ca2+-dependent (KCa5 activation = 1.0-1.5 mM Ca2+; Fig. 4A). The pH optimum of this conversion was acidic (pH 5.5, Fig. 4B); there was little proalbumin processing below pH 4.0 or above pH 7.0 (Fig. 4B). These observed Ca2+ and pH dependences of insulin secretory granule proalbumin processing activity are virtually identical to those of the type I proinsulin processing endopeptidase activity (20). Group-specific inhibitors of serine, cysteinyl, or aspartyl
Proalbumin Conversion by a Proinsulin Processing Endopeptidase

Fig. 3. Proprotein processing activity in fractions obtained from DE52 ion-exchange chromatography of insulin secretory granules. The chromatography, [3H]-proinsulin processing assay (over 3 h), and [125I]-proalbumin processing assay (over 2 h) were as described (“Experimental Procedures”). Panel A shows the A280 profile (A) and NaCl gradient applied (A); panel B shows the specific proinsulin processing in the fractions collected of type I (Arg⁴-Arg³-directed (U)) and type II (Lys⁴⁴-Arg⁵⁶-directed (M)) endopeptidases; panel C shows the Arg⁶-Arg⁶-directed proalbumin processing in the fractions from the same chromatographic run (C).

proteinases (26) had no effect on proalbumin processing by the insulin secretory granule fraction, whereas the metal ion chelators EDTA, CDTA, and EGTA, but not 1,10-phenanthroline, all inhibited this activity (Table I, Fig 1). The inhibition by EDTA was fully restored by the addition of Ca²⁺ (Fig. 2). No inhibitory effects of the epoxide inhibitors Ep-459, Ep-475, and E-64 (potent inhibitors of lysosomal cathepsins-B and -L (26)) or vanadate were observed (Table I). The proteinase active-site-directed inhibitor tosyl-L-lysyl-chloromethane or tosyl-L-phenylalanyllchloromethane had no effect on insulin secretory granule proalbumin processing activity. The corresponding dibasic tripeptide Ala-Lys-Arg-CH₂Cl, however, produced over 90% inhibition (Table I) whereas the monobasic chloromethane tripeptide Ala-Nle-Arg-CH₂Cl inhibited this conversion activity to a much lesser extent. This apparent preference for dibasic sequences was illustrated further in that the monobasic sequence inhibitors leupeptin and antipain were only partially inhibitory of millimolar concentrations (Table I). The dibasic sulfonium salt tripeptide Ala-Arg-Arg-CH₂S⁺(CH₃)₂, which contains the same dibasic sequence as proalbumin, inhibited proalbumin conversion at a lower concentration than Ala-Lys-Arg-CH₂S⁺(CH₃)₂. This reaffirms the Arg-Arg specificity of this processing endopeptidase activity inferred from the results obtained after DE52 chromatography (Fig. 3) (27).

The proalbumin processing by insulin secretory granules was inhibited by 20 mM F⁻ but not by Cl⁻, Br⁻, I⁻, SCN⁻, and PO₄⁻ at equivalent concentrations (Table I). The activity was inhibited by Zn²⁺ and to a lesser extent by Mn²⁺ and Co²⁺ (Table I). Similar inhibitory effects by these cations have been observed for insulin secretory granule proinsulin conversion (17).

Both the proinsulin and proalbumin converting activity of insulin secretory granules were inhibited by α₁-antitrypsin Pittsburgh (Fig. 5) over a similar concentration range (Kₐ₅₀ inhibition = 100 nM for proalbumin conversion and 126 nM for proinsulin conversion). Proinsulin conversion, like proal-
**TABLE I**

| Inhibitor                        | Control activity |
|----------------------------------|------------------|
| Phenylmethylsulfonyl fluoride (10) | 119.9            |
| Phenylmethanesulfonate fluoride (1) | 104.2            |
| 3,4-Dichloroisocoumarin (0.4)     | 98.8             |
| 2,2'-Dipyridyl disulfide (1)      | 102.7            |
| Tosyl-L-lysylchloromethane (1)    | 98.9             |
| Iodoacetic acid (1)               | 109.2            |
| Iodoacetamide (1)                 | 109.6            |
| N-Ethylmaleimide (1)              | 98.5             |
| p-Chloromercuribenzoate (0.1)      | 0                |
| Hg²⁺ (0.1)                       | 3.5              |
| EDTA (6)                         | 0                |
| CDTA (6)                         | 0                |
| EGTA (6)                         | 29.0             |
| 1,10-Phenanthroline (6)          | 90.0             |
| Leupeptin (2)                    | 62.1             |
| Leupeptin (0.2)                  | 103.7            |
| Antipain (3)                     | 39.4             |
| Vanadate (0.1)                   | 113.8            |
| Ep-459 (1)                       | 99.3             |
| Ep-475 (1)                       | 89.3             |
| Ala-Nle-Arg-CH₂Cl (0.1)          | 65.5             |
| Ala-Lys-Arg-CH₂Cl (0.1)          | 4.6              |
| Ala-Lys-Arg-CH₂S(CH₂)₂ (0.1)     | 52.7             |
| Ala-Lys-Arg-CH₂S(CH₂)₂ (0.01)    | 100.0            |
| Ala-Arg-Arg-CH₂Cl (0.1)          | 11.2             |
| Ala-Arg-Arg-CH₂S(CH₂)₂ (0.01)    | 60.6             |
| Ala-Arg-Arg-CH₂S(CH₂)₂ (0.001)   | 105.9            |
| NaF (20)                         | 8.5              |
| ZnCl₂ (1)                        | 9.3              |
| MnCl₂ (1)                        | 56.1             |
| CoCl₂ (1)                        | 35.7             |

**FIG. 5.** The effect of α₁-antitrypsin Pittsburgh on proalbumin and proinsulin processing by insulin secretory granule endopeptidases. [³⁵S]Proprotein processing was assayed as described (see "Experimental Procedures") over a period of 3 h at 30 °C. Panel A shows [³⁵S]-proalbumin conversion in the presence of increasing concentrations of α₁-antitrypsin Pittsburgh (control activity = 66.7% proalbumin converted in the absence of inhibitor). Panel B shows [³⁵S]-proinsulin conversion in the presence of increasing concentrations of α₁-antitrypsin Pittsburgh (control activity = 48.8% proinsulin converted in the absence of inhibitor).

**DISCUSSION**

A variety of plasma membrane and secreted proteins, in many different cell types, are synthesized as proprotein precursors which are subsequently processed intracellularly at sequences marked by pairs of basic amino acids (I–3). The conservation of the post-translational proprotein proteolytic conversion mechanisms in eukaryotes has been inferred indirectly from a number of transfection studies. It appears to be neither a species-, cell type-, nor proprotein-specific process. Human proinsulin cDNA transfected into mouse pituitary cells (AtT20 cell line), which normally produce ACTH from the precursor proopiomelanocortin, transiently expresses proinsulin and correctly processes it to insulin (28). In similar experiments it has been found that proenkephalin can be processed to Met-enkephalin in AtT20 cells (29), and human preproparathyroid hormone is converted to parathyroid hormone in a rat pituitary cell line (GH₃ cells) (30). The proteolytic conversion of heterologous proteins is not confined solely to cells with a regulated secretory pathway. Other transfection experiments have shown preproparathyroid hormone conversion to parathyroid hormone in constitutively secreting mouse fibroblasts (NIH 3T3 cells) (30) and preprosomatostatin conversion to somatostatin-14 in monkey kidney fibroblasts (COS7 cells) (31). Further evidence for the conservation of the proprotein processing endopeptidase is seen in the observations that the yeast KEX-2 gene product, which normally cleaves pro-α-mating factor, also cleaves proinsulin (32), proalbumin (33), and proopiomelanocortin (34).

In this in vitro study, we demonstrate that a proinsulin processing endopeptidase activity of insulin secretory granules can also process proalbumin to albumin. This proalbumin processing endopeptidase activity was mainly attributed (>90%) to the Arg-Arg-specific type I proinsulin processing endopeptidase (20). The specificity of proalbumin conversion was confirmed by the ability of the reaction product to bind ⁶⁵Ni (32,35). Cleavage before or within the Arg⁻²⁻Arg⁻³ dibasic site and subsequent NH₂-terminal trimming are excluded as albumin production would then require the additional involvement of an aminopeptidase which would have been inhibited by 1,10-phenanthroline (Table I). The proalbumin processing activity of insulin secretory granules has almost identical biochemical characteristics to the Ca²⁺-dependent proalbumin conversion endopeptidase present in liver membrane preparations (11). Both these activities have the same Ca²⁺ and pH requirements, the same dibasic specificity, will not process the proalbumin Christchurch mutant (22), are insensitive to the same group-specific proteinase inhibitors, and are inhibited by the α₁-antitrypsin Pittsburgh mutant (21,36). Proalbumin conversion in hepatocytes and proinsulin conversion in the pancreatic β-cell therefore appear to be cleaved by very similar Arg-Arg-directed endopeptidase activities.

The major intracellular site of proinsulin conversion is the secretory granule (12–18), which with its acidic intragranular pH (5–6) (12, 24) and its free Ca²⁺ concentration of 1–10 mM (25) constitutes an optimal environment for the proinsulin processing endopeptidase types I and II (20). The constitutive transport vesicle, involved in proalbumin processing (4, 5, 7) has an acidic inner environment (37), and one would predict that on the basis of our present findings the intravesicular Ca²⁺ concentration is in the millimolar range. The formation of a secretary granule in pancreatic β-cells or of a constitutive transport vesicle in hepatocytes occurs in the region of the trans-Golgi network (4, 5, 7, 12). It is generally believed that
this particular compartment has a relatively low Ca\(^{2+}\) concentration (98) and a near neutral pH (12). It follows, therefore, that the formation of either a secretory granule or constitutive transport vesicle is accompanied by the insertion or activation of proteins which result in intragranular acidification and Ca\(^{2+}\) accumulation. This common feature, together with the findings that proprotein processing occurs by a similar enzymic activity in both the constitutive and regulated secretory pathways, suggests that these compartments contain many molecular components in common.

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SUPPLEMENTARY MATERIAL TO:

Proalbumin to Albumin Conversion by a Proinsulin Processing Endopeptidase of Insulin Secretory Granules.

Christopher J. Rhodes, Stephen O. Brennan and John C. Hutton.

EXPERIMENTAL PROCEDURES

Materials

Human proalbumin and ox-α-propionyl-heptanoyl-vallile-arginyl-arginyl-lysine (ox-α-PAR) were isolated from the plasma of a normal child (98). The proalbumin ox-PAR variant was isolated from the plasma of a hemoglobinopathie (98) and ox-α-PAR was isolated from the plasma of a patient with previously described (99) Human monos (99). Proinsulin was a gift from Dr. Bruce Shaw (Shaw Institute, Basel, Switzerland). Unless indicated otherwise, all other chemicals were purchased from Sigma (London) Chemical Company, Poole, Dorset, U.K. or BDH, Poole, Dorset, U.K., and were of the highest grade or purity available.

Tissue

Isolated islets of Langerhans (98, 99), prepared as described (98), were used as a source of purified β-cells and for the pericellular 5′-nucleotidase.

Tissue culture

Isolated islets were used as a source of purified β-cells and for the pericellular 5′-nucleotidase.

Experimental conditions

Suspension of type I and II pro-insulin processing endopeptidases was achieved by an exchange chromatography of a 6-glycine fraction on DEAE-cellulose (20). In brief, 8

Proalbumin Conversion by a Proinsulin Processing Endopeptidase of Insulin Secretory Granules.
Proalbumin Conversion by a Proinsulin Processing Endopeptidase

Proalbumin conversion assay

Unless otherwise stated, aliquots of a purified 5-guinea-fraction (90% protein) or DE-52 column fractions were incubated for 0 to 96 hours at 37°C in 0.1 M (final volume) of 0.1% (w/v) BSA 100 mM sodium acetate, 100 mM NaCl, 10 mM Tris, 10 mM E-64, pH 8.6, containing 25,000 cpm of [35S]proalbumin at specific activity 330 Ci/mmol, as previously described [17,20]. The [35S]proalbumin generated was analyzed by alkali-urea gel electrophoresis and autoradiography [17].

In a 1 h incubation between 50 and 60% [35S]proalbumin was processed to insulin and conversion intermediates (des 31-33 proinsulin and des 44-65 proinsulin) by a lysed secretion culture fraction. The [35S]proalbumin was assayed to either 10% to des 31-33 proinsulin by the DE-52 separated Arg, Arg-directed type endopeptidase or 15% and 10% [35S]labeled des 44-65 proinsulin (13-19), des 31-32 proinsulin and insulin (18-36) with the lysed Arg-directed type endopeptidase, in a similar 3 h period (Figure 2) [17,20].

Proalbumin converting assay

[35S]proalbumin was prepared by the chloramine T technique [21]. Proalbumin processing activity was assayed by including aliquots of a purified 5-guinea-fraction (90% protein) or DE-52 column fractions for 1-6 h at 37°C in 0.1 M Tris, 100 mM sodium acetate, 100 mM NaCl, 10 mM Tris, 10 mM E-64, pH 8.6, containing 25,000 cpm of [35S]proalbumin at specific activity 330 Ci/mmol. The incubation was stopped by the addition of type I gelatin by the addition of 0.5 M sodium borate buffer (pH 8.6). Samples (10 µl) were subjected to electrophoresis in a 15% (w/v) gel at 350 V. After electrophoresis the gel was dried, autoradiographed (48 h), fixed, and stained.

After a 3 h incubation with insulin secretion culture extract, between 50-60% of [35S]proalbumin was quantitatively converted to albumin (Figure 4). Conversion was linear up to 3 h and about 90-90% of the proalbumin had been converted by 6 h. An assay period of 2-3 h was generally used to ensure proalbumin conversion was quantitatively assessed over a linear segment of the time course. The assay was carried out in the presence of 0.25 M ethylenediamine tetraacetate (EDTA) and 10 mM E-64 to inhibit any nonspecific proteolysis. Note that these peptidase inhibitors had any specific effect on proalbumin or proinsulin conversion [17,20] activity by insulin secretion culture factors.

Other procedures

Protein was determined by the method of Bradford [42] using bovine serum albumin as a standard. Insulin content of subcellular fractions was quantified by radioimmunoassay [14]. Free Cu2+ concentration was calculated from electron spectroscopies as previously described [45].

Figure M1. Time course of [35S]proalbumin conversion to [35S]albumin by an insulin secretion culture endopeptidase. Proalbumin conversion was assessed as described (Methods section), and expressed as the percentage of the original proalbumin radioactivity recovered in albumin, by 9.91 (± 0.8 %) of the proalbumin had been converted. The results shown are the mean ± SEM of at least three independent observations.

granules (10-30 mg protein) were sonicated (15 s, 4°C, 35 wpp) in 4.5 ml of 20 mM bis-Tris, 0.5% Triton X-100, 100 mM sodium phosphorlase (NADH), 100 mM sodium EDTA, 0.1 M NaOH in 0.1 M saccharose, 10 mM phosophorylase A, pH 6.5. The reaction mixture was then centrifuged at 17,000 x g for 5 min. 4°C, 100.000 g and the supernatant applied at 0.5 mmol of 8 to 40 ml DE-52 column (Wakamatsu, Ltd., Wakamatsu, Ky., U.S. equilibrated in 200 mM bis-Tris buffer pH 6.5. The column was washed with 27.5 ml of the same equilibration buffer, then eluted with a 30 ml linear gradient of 0.04-0.4 M NaCl gradient in the same buffer. Fractions (1 ml) were collected, and the buffer in each of these was immediately exchanged with 10.0 M sodium acetate (pH 5.5) over a dialyzing Sephadex G-25 spacer column (1 ml). Each fraction was then assayed for proalbumin and proalbumin processing activity.

Proalbumin conversion assay

Unless otherwise stated, aliquots of a purified 5-guinea-fraction (90% protein) or DE-52 column fractions were incubated for 0 to 96 hours at 37°C in 0.1 M (final volume) of 0.1% (w/v) BSA 100 mM sodium acetate, 100 mM NaCl, 10 mM Tris, 10 mM E-64, pH 8.6, containing 25,000 cpm of [35S]proalbumin at specific activity 330 Ci/mmol, as previously described [17,20]. The [35S]proalbumin generated was analyzed by alkali-urea gel electrophoresis and autoradiography [17].

In a 1 h incubation between 50-60% [35S]proalbumin was processed to insulin and conversion intermediates (des 31-33 proinsulin and des 44-65 proinsulin) by a lysed secretion culture fraction. The [35S]proalbumin was assayed to either 10% to des 31-33 proalbumin by the DE-52 separated Arg, Arg-directed type endopeptidase or 15% and 10% [35S]labeled des 44-65 proalbumin (13-19), des 31-32 proalbumin and insulin (18-36) with the lysed Arg-directed type endopeptidase, in a similar 3 h period (Figure 2) [17,20].

Proalbumin converting assay

[35S]proalbumin was prepared by the chloramine T technique [21]. Proalbumin processing activity was assayed by including aliquots of a purified 5-guinea-fraction (90% protein) or DE-52 column fractions for 1-6 h at 37°C in 0.1 M Tris, 100 mM sodium acetate, 100 mM NaCl, 10 mM Tris, 10 mM E-64, pH 8.6, containing 25,000 cpm of [35S]proalbumin at specific activity 330 Ci/mmol. The incubation was stopped by the addition of type I gelatin by the addition of 0.5 M sodium borate buffer (pH 8.6). Samples (10 µl) were subjected to electrophoresis in a 15% (w/v) gel at 350 V. After electrophoresis the gel was dried, autoradiographed (48 h), fixed, and stained.

After a 3 h incubation with insulin secretion culture extract, between 50-60% of [35S]proalbumin was quantitatively converted to albumin (Figure 4). Conversion was linear up to 3 h and about 90-90% of the proalbumin had been converted by 6 h. An assay period of 2-3 h was generally used to ensure proalbumin conversion was quantitatively assessed over a linear segment of the time course. The assay was carried out in the presence of 0.25 M ethylenediamine tetraacetate (EDTA) and 10 mM E-64 to inhibit any nonspecific proteolysis. Note that these peptidase inhibitors had any specific effect on proalbumin or proinsulin conversion [17,20] activity by insulin secretion culture factors.

Other procedures

Protein was determined by the method of Bradford [42] using bovine serum albumin as a standard. Insulin content of subcellular fractions was quantified by radioimmunoassay [14]. Free Cu2+ concentration was calculated from electron spectroscopies as previously described [45].

Figure M1. Time course of [35S]proalbumin conversion to [35S]albumin by an insulin secretion culture endopeptidase. Proalbumin conversion was assessed as described (Methods section), and expressed as the percentage of the original proalbumin radioactivity recovered in albumin, by 9.91 (± 0.8 %) of the proalbumin had been converted. The results shown are the mean ± SEM of at least three independent observations.