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STUDIES ON THE RELATIVE RATES OF INTRACELLULAR TRANSPORT OF EGG WHITE PROTEINS

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Summary

In order to determine if the rates of intracellular transport of oviduct secretory proteins are coordinate, oviduct minces were first incubated with either [^{3}H]methionine or [^{3}H]leucine. 2 h later, the minces were exposed to either [^{35}S]methionine or [^{14}C]leucine in addition to the ^{3}H-labeled amino acid. After incubating for an additional 90—120 min, aliquots of the media were subjected to immunoprecipitation to isolate ovalbumin, conalbumin and lysozyme. From the ^{3}H/^{35}S or ^{3}H/^{14}C ratios in the isolated proteins, relative rates of intracellular transport were determined. The data obtained indicate that conalbumin and lysozyme exhibit similar rates of intracellular transport, whereas ovalbumin lags behind these proteins considerably. The basis for this effect is discussed in light of current knowledge concerning the synthesis and secretion of ovalbumin.

Introduction

It is now well established that proteins destined for export from the cell follow a common pathway in which they are synthesized on membrane-bound ribosomes, discharged into the lumen of the endoplasmic reticulum, and packaged into secretory granules in the Golgi complex (see Ref. 1 for a review). A major question concerning this scheme is whether all secretory proteins in a given cell are processed in a parallel fashion. In an elegant study in guinea pig pancreas, Tartakoff et al. [2] demonstrated that, within the resolution of the methodology employed, the major pancreatic secretory proteins enter the condensing vesicles of the Golgi complex and appear in zymogen granules in appar-

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.

ent synchrony. However, parallelism in transport from the site of synthesis in the endoplasmic reticulum was not critically investigated.

There are several reports which suggest that, in some cases, secretory proteins are not processed in parallel. Keller et al. [3] found that the appearance of radioactive ribonuclease in pancreatic juice lagged behind that of chymotrypsinogen and trypsinogen following in vivo labeling with [^{14}C]arginine. Similar results were found by Christophe et al. [4] in studies of lipase secretion from pancreas. In addition, Morgan and Peters [5] and, more recently, Schreiber et al. [6] presented convincing evidence that transferrin secretion from rat liver requires significantly more time than does albumin secretion. Morgan and Peters speculated that, since transferrin is a glycoprotein whereas albumin is not, the increased time may be due to the addition of carbohydrate residues.

Work in this laboratory has been concerned with determining the effect, if any, of post-translational modifications on the intracellular transport and secretion of egg white proteins from hen oviduct. The oviduct secretory proteins which we chose to investigate were ovalbumin, conalbumin and lysozyme. These proteins are known to undergo a variety of modifications during and after synthesis. Ovalbumin is both glycosylated (one oligosaccharide chain) and phosphorylated (one or two residues) [7]; conalbumin is also glycosylated (one chain) [8] and is synthesized with an N-terminal signal sequence which is removed during translation [9]. Lysozyme, which lacks carbohydrate, also has a signal sequence removed during synthesis [10].

The results of the present studies suggest that the times required for the intracellular transport of these three proteins from the tubular gland cell of the oviduct are not identical, and raise the possibility that post-translational modifications may influence their rate of intracellular transit.

Materials and methods

[4,5-^{3}H]Leucine (35 Ci/mmol), [U-^{14}C]leucine (312 Ci/mol), and [*methyl*-^{3}H]methionine (13 Ci/mmol) were from ICN. [^{35}S]Methionine (407 Ci/mmol) was from New England Nuclear.

Assay of secretion rates. Oviducts were removed from actively laying white leghorn hens, and small minces (approx. 20 mg each) were prepared from the magnum region using sterile scalpels. The tissue (300 mg total weight) was placed in 3 ml Hank's salt solution containing Hepes (20 mM, pH 7.4), penicillin (100 units/ml), streptomycin (100 μg/ml), 19 unlabeled amino acids (1 mM each) and a single labeled amino acid. The tissue was incubated at 37°C in 10-ml Ehrlenmeyer flasks in a shaking incubator at 120 cycles per minute. The flasks were constantly flushed with a stream of humidified oxygen. Labeled secreted protein was monitored using gel filtration on Sephadex G-25 according to Keller and Swank [11].

To determine the relative rates of secretion of particular proteins, a double label protocol was followed. Minces were first incubated for 2 h with a ^{3}H-labeled amino acid (200 μCi, methionine or leucine). The media was then removed and replaced with fresh media containing the same ^{3}H-labeled amino acid (200 μCi) plus 20 μCi of either [^{35}S]methionine (when [^{3}H]methionine was used in the first incubation) or [^{14}C]leucine (when [^{3}H]leucine was used).

It was necessary to change the medium after the first incubation because a large quantity of adhered egg white protein is washed off the minces during the first incubation. This protein would have compelled the use of large amounts of antibodies and resulted in immunoprecipitates too concentrated for gel electrophoretic analysis. In control experiments, minces were exposed to 1 mM of all 20 amino acids in the first incubation. At the indicated times, aliquots of the medium were removed and centrifuged briefly at a low speed to remove debris. The entire supernatant was then applied to a Sephadex G-25 column (1 × 20 cm) equilibrated with distilled water. The void volume fractions containing the secreted protein were pooled, lyophylized and taken up in 0.5 ml 0.2 M NaCl. Aliquots of this material were than subjected to immunoprecipitation with specific antibodies which had been purified on antigen-Sepharose columns. Since ovalbumin, conalbumin and lysozyme are present in different proportions in the secretory fluid of the oviduct, different size aliquots were taken for immunoprecipitation of these proteins. Thus, only 20 μl were used for ovalbumin precipitation, whereas 100 μl and 300 μl were used for the immunoprecipitation of conalbumin and lysozyme, respectively. Immunoprecipitates were treated according to Palmiter et al. [12] and subjected to polyacrylamide slab gel electrophoresis in SDS according to Laemmli [13]. 2-mm slices of the gels were prepared and incubated overnight at 37°C in 0.5 ml 1% SDS. Following incubation, 5 ml of Aquasol (New England Nuclear) were added, the vials were vortexed vigorously, and the gel slices removed. The vials were then counted in a Packard liquid scintillation counter using a double label program in which the efficiency of ^{3}H and either ^{35}S or ^{14}C was 19% and 65%, respectively. The spillover of ^{14}C or ^{35}S cpm into the ^{3}H channel was 10%.

Results

Secretion of oviduct proteins in vitro. When oviduct minces are incubated with labeled amino acids, radioactive protein begins accumulating in the tissue immediately and continues in a linear fashion for at least 3 h [11]. After approximately 60 min, labeled protein can be detected in the medium. The amount of label secreted into the medium is quite small (approx. 5%) relative to the total labeled protein in the tissue, suggesting that the newly synthesized protein is diluted into a large pool of unlabeled secretory protein. Efforts to increase the amount of protein secreted using a variety of known secretogogues and other compounds (carbamylcholine (100 μM), isoproterenol (10 μM), diethylstilbesterol (0.03 μg/ml), norepinephrine (100 μM), dibutyryl cyclic AMP (1 mM), prostaglandins $F_{2\alpha}$ and E (both at 5 μg/ml)) were unsuccessful. These results are consistent with the hypothesis that egg white protein secretion is induced by the mechanical pressure of the egg yolk against the surface of the oviduct, rather than by a chemical stimulus (see Ref. 14 for a discussion of this subject).

Determination of relative rates of intracellular transport. Since we were interested in determining the relative rates of intracellular transport of ovalbumin, conalbumin and lysozyme, a double label protocol was carried out. Oviduct minces were first exposed to [^{3}H]methionine or [^{3}H]leucine for 120 min, after which time labeled protein was easily detectable in the medium. The medium was then changed and replaced by medium containing the ^{3}H-labeled

amino acid and either [^{35}S]methionine (when [^{3}H]methionine was used in the first incubation) or [^{14}C]leucine (when [^{3}H]leucine was used). As shown in Fig. 1 for the case of methionine labeling, ^{3}H cpm began to rise immediately after the beginning of the second incubation. As expected, protein-bound ^{35}S cpm did not appear in the medium until the 60 min time point.

If the rates of transit through the cell of the three egg white proteins are different, then any protein which exits after another will exhibit a higher ^{3}H/^{35}S ratio due to the fact that the tissue will have secreted a lesser amount of the ^{35}S-labeled slower-moving protein. Furthermore, the difference in ^{3}H/^{35}S ratios will be greatest at early time points and tend to decrease as time proceeds. To determine the ^{3}H/^{35}S ratio for each protein, aliquots of the medium, which had previously been subjected to gel filtration to remove free amino acids, were treated with specific antibodies and the resulting immunoprecipitates analyzed by gel electrophoresis in SDS. As shown in Fig. 2 the immunoprecipitates were

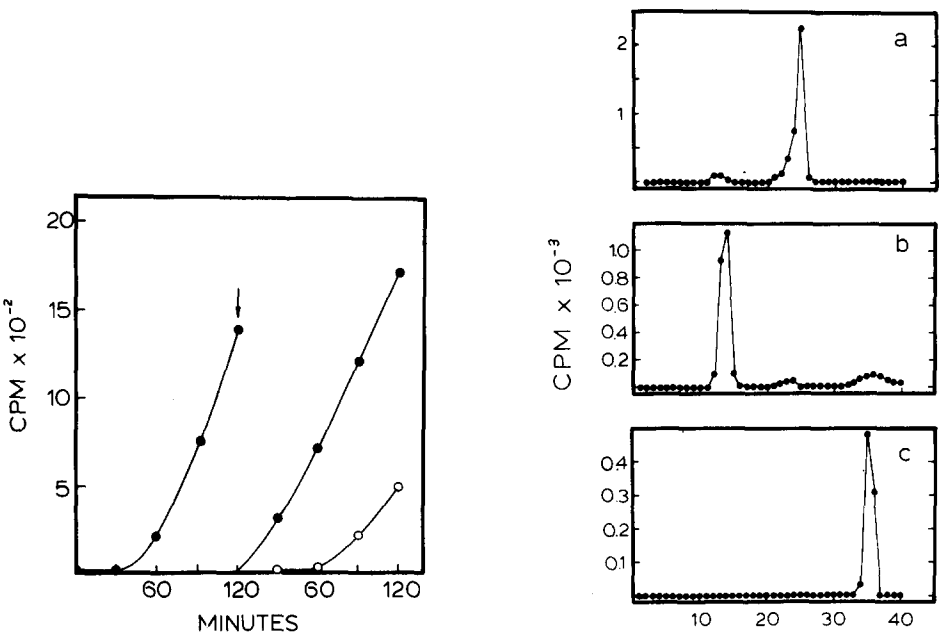

Fig. 1. In vitro secretion of egg white proteins using double label protocol. Oviduct minces were incubated as described in the text in the presence of [^{3}H]methionine (200 μCi). Secreted protein was determined by carrying out gel filtration (Sephadex G-25) on an aliquot (25 μl) of the medium [11]. After 120 min (arrow) the medium was changed and replaced with fresh medium containing [^{3}H]methionine (200 μCi) and [^{35}S]methionine (20 μCi). Aliquots were taken at the indicated time points and processes as before. All radioactivity measurements were carried out using a double label program. (●———●), Protein-bound ^{3}H cpm; (○———○), protein-bound ^{35}S cpm.

Fig. 2. Typical gel electrophoresis profiles of antibody-precipitated proteins. Oviduct minces were incubated with [^{3}H]methionine for 120 min and then incubated again with [^{3}H]methionine in fresh medium ([^{35}S]methionine was not added in this experiment). After 120 min of the second incubation, aliquots of the secreted protein fraction obtained by gel filtration off the medium were subjected to immunoprecipitation. The precipitates were dissolved in electrophoresis sample buffer, loaded onto a polyacrylamide slab gel and electrophoresed in SDS according to Laemmli [13]. Slices (2 mM) were prepared and counted as described in Materials and Methods. Profiles obtained from immunoprecipitated ovalbumin (a); conalbumin (b); lysozyme (c).

free of contaminating proteins. The gel fractions containing the purified proteins were counted to 1% accuracy and the ^{3}H dpm/^{35}S dpm was computed. As shown in Experiment I of Table I, the $^{3}H/^{35}S$ ratio for ovalbumin is considerably greater than that for conalbumin or lysozyme, suggesting that ovalbumin requires a longer time to exit from the cell. Also, the difference in $^{3}H/^{35}S$ ratios declines with time, (compare 90 min and 120 min ratios).

If the data obtained with [^{3}H]- and [^{35}S]methionine is valid, it should be independent of the amino acid employed. As shown in experiment III of Table I, when [^{3}H]- and [^{14}C]leucine were used as the labels, essentially identical results were obtained, i.e., ovalbumin had a substantially greater $^{3}H/^{14}C$ ratio than conalbumin or lysozyme.

Experiments II and IV of Table I are controls in which either [^{3}H]- and [^{35}S]methionine (II) or [^{3}H]- and [^{14}C]leucine (IV) were added simultaneously after a 120 min preincubation with unlabeled amino acid. The data show no major difference in the ratios for ovalbumin and conalbumin in these experiments, indicating that the differences observed in experiments I and III were not due to artifacts caused by the double label protocol.

In order to investigate the possibility of differential metabolism of ^{3}H and

TABLE I

DETERMINATION OF RELATIVE RATES OF SECRETION OF EGG WHITE PROTEINS USING DOUBLE LABEL PROCEDURE

Oviduct minces were first incubated for 120 min with labeled amino acids (Expts. I and III) or unlabeled amino acids (control Expts. II and IV) as described in the text. The medium was then removed and fresh medium containing ^{3}H- and either ^{35}S- or ^{14}C-labeled amino acids was added and the minces reincubated. Relative secretion rates were determined by immunoprecipitation from the medium after removal of free amino acids by gel filtration. Immunoprecipitates were analyzed on SDS-polyacrylamide gels and the fractions containing the labeled proteins identified and counted to 1% accuracy. The amino acid precursor refers to the labeled amino acids which were added to the media in the second incubation. All values of radioactivity incorporated are corrected to dpm using an external standard. ova, ovalbumin; con, conalbumin; lys, lysozyme.

Expt.	Amino acid precursor	Time (min)	Protein	Radioactivity incorporated		Ratio $^{3}H/^{35}S$
				^{3}H	^{35}S	
I	[^{3}H]- + [^{35}S]Met	90	ova	5270	130	40
			con	4890	230	21
			lys	1860	81	23
		120	ova	12800	631	20 (27 *)
			con	6490	430	15 (18 *)
			lys	1820	117	16 (18 *)
II	[^{3}H]- + [^{35}S]Met	90	ova	580	127	4.6
			con	1010	216	4.7
				^{3}H	^{14}C	$^{3}H/^{14}C$
III	[^{3}H]- + [^{14}C]Leu	120	ova	2710	150	18
			con	3640	288	13
			lys	3970	298	13
IV	[^{3}H]- + [^{14}C]Leu	120	ova	1440	131	11
			con	1580	144	11

* These ratios have been corrected for the removal of half the medium at the 90 min time point.

^{35}S in the case of methionine or ^{3}H and ^{14}C in the case of leucine, amino acid analyses were carried out. After the completion of the secretion experiments, homogenates of the tissue were prepared in distilled water and centrifuged at 100 000 × *g*. The resulting supernatants were passed over Sephadex G-25 and the protein-containing fractions subjected to acid hydrolysis (6 N HCl, 110°C, 48 h for leucine; performic acid treatment according to Hirs [15] followed by acid hydrolysis as above for methionine). Thin-layer chromatography of the hydrolysates indicated that approx. 90% of the ^{3}H and ^{35}S comigrated with methionine sulfone and 99% of the ^{3}H and ^{14}C comigrated with leucine. Thus, there does not appear to be substantial metabolism of the labeled amino acids during the incubations.

Discussion

There have been several investigations in recent years concerning the kinetics of intracellular transport and secretion [2—6]. Most of these studies were carried out using a single radioisotope to follow intracellular transport. In the present study we have employed a double label technique to follow the relative rates of intracellular transport of egg white proteins. The double label protocol has two important advantages. First, it eliminates the problem of quantitative recoveries of the proteins, since only the ratio of the isotopes is relevant. Second, only one time point need be taken, although, because the addition of the isotopes is staggered, the ratio of the isotopes in a given secreted protein sample will decrease as a function of time. Therefore, early time points will show the maximum difference in rates of intracellular transport.

The results obtained in this investigation strongly suggest that the total time required for the synthesis, transport, and secretion of ovalbumin from the tubular gland cell is significantly greater than that for conalbumin or lysozyme. An alternative explanation is that the newly synthesized proteins are diluted into pools which are disproportionate to their rates of synthesis. Thus, ovalbumin, which is approximately 55% of total protein synthesized, could be diluted into a pool which represents greater than 55% of the intracellular secreted protein. This would result in a lag in the appearance of radioactive ovalbumin because of the disproportionate dilution of this protein. This possibility appears highly unlikely in view of the fact that Edwards et al. [22] have shown that the levels of the egg white proteins in the magnum are proportional to their rates of synthesis and relative abundance in the egg. A second alternative explanation is that the relative rates of synthesis of the egg white proteins change during the course of incubation. Thus, if the rate of ovalbumin synthesis declined during the second incubation, a lag in the appearance of the second isotope in ovalbumin would be observed. Although this possibility cannot be completely ruled out, previous work by us [11] and Palmiter et al. [12] indicates that the rate of total oviduct protein synthesis continues linearly for several hours under tissue mince conditions.

Because of the non-linearity of the secretion curve (Fig. 1), it is difficult to estimate the additional time required for ovalbumin secretion. A rough estimate, based on the data of Table I and the fact that ovalbumin secretion in vitro requires 55 min [11], can be obtained graphically (not shown) and yields a value of approximately 10 min.

There are several possible mechanisms which would explain the increased time for ovalbumin transit. First, the rate of elongation of the polypeptide chain could be slower for ovalbumin than conalbumin or lysozyme. This possibility seems unlikely in view of the results of Palmiter [16] and unpublished work in our laboratory which demonstrates that the ribosomal transit time for ovalbumin, conalbumin and lysozyme are proportional to the molecular weights of these proteins. A second possibility is that the post-translational modifications which ovalbumin undergoes are rate-limiting in intracellular transport. This is probably not the case with respect to glycosylation, since we have shown [11] that the time required for secretion of unglycosylated ovalbumin in the presence of tunicamycin is the same as native ovalbumin. Ovalbumin does undergo phosphorylation, however, and this modification could be a rate-limiting step. Along these lines, it would be interesting to examine the time course of secretion of vitellogenin, a heavily phosphorylated glycoprotein secreted from the liver of female oviparous vertebrates [17], A third possible explanation involves the means by which ovalbumin is transported into the lumen of the endoplasmic reticulum. Lingappa et al. [18] have recently shown that the signal sequence for ovalbumin is localized in the interior of the protein molecular rather than the amino terminus, as has been shown for a wide variety of other proteins [19], including conalbumin and lysozyme [9,10]. Furthermore, these investigators found that the internally located signal (residues 234—253) functions in vitro to initiate translocation of the protein into the lumen of the endoplasmic reticulum. Assuming a polypeptide chain elongation rate of 200 amino acids per min at 37°C (see Palmiter [16]) the transport of ovalbumin into the endoplasmic reticulum could be delayed by approx. 1.2 min (234 amino acids divided by 200 amino acids polymerized per min). Such an effect could partially explain the lag in intracellular transport we have observed. However, Meek et al. [23] have recently presented preliminary evidence which contradicts the work of Lingappa et al. [18]. A fourth possibility is that the secretion granules of the tubular gland cell are heterogeneous and that ovalbumin is packaged into a pool of slowly discharging granules. Heterogeneity in the granule content of polymorphonuclear granulocytes has been reported by Bainton and Farquhar [21]. However, as these authors point out, the two types of granules that were distinguished were probably secretory vesicles and lysosomes. In the case of the tubular gland cells of the oviduct, there is no evidence to suggest heterogeneity in the composition of the secretory granules, nor are there teleological reasons to expect it.

It should be emphasized that the system of monitoring egg white protein secretion which we have employed in the present study is necessarily non-physiological. In vivo, the oviduct continuously synthesizes egg white proteins and transports them to the secretory granules for storage. Secretion of the bulk of the granule contents takes place over a short period of time (approx. 2 h for the entire 30 cm magnum) and is probably triggered by the mechanical pressure of the yolk against the tubular gland cells of the magnum. Therefore, the secretion we observe in vitro is probably basal, or low level 'leakage' of stimulus-induced secretion. Despite this fact, we feel that the results obtained are valid, since it would appear unlikely that the amount of protein secreted could influence the relative rates of intracellular transport of the proteins. Neverthe-

less, it would be desirable to verify the present in vitro results in vivo. Unfortunately, it would be difficult to detect the small differences in transit times since the proteins are stored at least 20 h after synthesis before being secreted [22].

The data obtained in the present report, coupled with previous work from several laboratories [3—6,20] strongly suggest that secretory proteins are not necessarily transported through the cell at identical velocities. This finding, therefore, raises the possibility that certain co-translational or post-translational modifications may be rate-limiting in intracellular transport.

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