Membrane Alteration Is Necessary but Not Sufficient for Effective Glutamate Secretion in *Corynebacterium glutamicum*

CHRISTIAN HOISCHEN AND REINHARD KRÄMER*

Institut für Biotechnologie I der Kernforschungsanlage Jülich, 5170 Jülich, Federal Republic of Germany

We showed recently that secretion of glutamate in biotin-limited cells of *Corynebacterium glutamicum* is mediated by carrier systems in the plasma membrane (C. Hoischen and R. Krämer, Arch. Microbiol. 151:342–347, 1989). In view of the generally accepted hypothesis that glutamate efflux is directly caused by alterations of the membrane, it was necessary to examine the kind of correlation between changes in lipid content and composition of the bacterial membrane and glutamate secretion activity. Two new experimental approaches were used. (i) Changes in lipid content and composition were analyzed in glutamate-producing cells which were forced to switch to nonproducers by addition of biotin in a short-term fermentation. (ii) The time courses of both the fatty acid or phospholipid composition and the efflux activity were analyzed within the first minutes of the switch from high to low secretion activity. The following results were obtained. (i) The time course of the change in fatty acid or phospholipid content and composition was not related to the change in secretion behavior. (ii) There was no specific fatty acid or phospholipid compound which regulated glutamate efflux. (iii) High efflux activity could only be induced when the total lipid content of the membrane was reduced. (iv) Although consistently correlated to high secretion activity, membrane alteration was never a sufficient prerequisite for glutamate efflux in *C. glutamicum*.

Corynebacterium glutamicum and other coryneform bacteria have been widely used for the production of glutamate and a number of other amino acids for more than 30 years. Glutamate secretion can be induced by various treatments, such as biotin limitation (41), addition of specific fatty acid derivatives (44), addition of penicillin (32), or fermentation, using oleic acid (23) or glycerol (18, 31), auxotrophs. All of these treatments are obviously related to alterations of the bacterial membrane. In this paper, we focus on the most intensely investigated procedure, i.e., growth under biotin limitation.

Mainly, two controversial models have been favored to explain the mechanism of amino acid secretion in general and the secretion of glutamate in particular. The generally accepted hypothesis for glutamate secretion under biotin limitation ("leak model") is based on the observation that lack of biotin decreases fatty acid synthesis, causing alterations in the plasma membrane, i.e., decreasing the lipid content and changing the phospholipid and fatty acid composition (45). Thus, the plasma membrane is postulated to become permeable to glutamate, leading to secretion by passive diffusion (4, 9, 21, 22, 30, 39–41). The internal concentration of glutamate then decreases to such an extent that the feedback inhibition of glutamate synthesis is abolished. The second hypothesis is based on the general model for many efflux processes in bacteria ("inversion model"), i.e., inversion of the corresponding uptake system (6, 7, 28, 29, 35). The glutamate uptake carrier is supposed to be functionally uncoupled due to a decrease in membrane phospholipids.

In a previous paper (14), we presented data which rule out the leak model for glutamate secretion and render the inversion model very unlikely. Based on results concerning specificity and kinetics of glutamate transport as well as efflux regulation, the secretion of glutamate in *C. glutamicum* was shown to be mediated by a special efflux carrier system. Also, isoleucine secretion by *C. glutamicum* was demonstrated to be catalyzed by a specific secretion carrier (11). It should also be mentioned that efflux of 5'-IMP (46, 47) and NAD (36) in *Brevibacterium ammoniagenes* was shown not to be mediated by passive efflux.

We are aware of the fact that the various treatments leading to glutamate secretion are different from those used for inducing efflux of other amino acids. In the latter cases, basically no alteration of the bacterial membrane has been observed. As mentioned above, induction of glutamate secretion obviously affects the physical state of the plasma membrane (15, 19, 20, 26, 38, 45). The kind of correlation, however, between the carrier-mediated efflux and the observed membrane changes is unknown. Although repeatedly stated, it has not been shown that membrane alterations and efflux activity are, in fact, causally related or whether they are only temporally connected. Even if one assumes that alterations of the membrane caused by biotin limitation are essential for glutamate efflux, it is not clear whether these changes are the only and sufficient prerequisite for glutamate efflux. When taking into consideration other biotin-dependent enzymes of the cytosol (25) or recently discovered biotin-dependent transport systems (10), it is not even clear whether the influence of biotin is solely explained by its effect on fatty acid synthesis.

The main reason for the lack of unequivocal data in this respect is that, in studies published so far, two completely different cell populations, i.e. biotin-limited producer cells and biotin-supplemented "normal" cells, have been compared. By using short-term fermentation (14), we succeeded in direct and controlled interconversion of producing and nonproducing cells. In these experiments, the immediate changes in the lipid content and composition after inhibition of glutamate production could be directly compared with the corresponding effects on amino acid efflux. Thereby, we demonstrate that alterations in the lipid state of the membrane are necessary but not sufficient for inducing glutamate efflux in *C. glutamicum*.

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* Corresponding author.

MATERIALS AND METHODS

Organisms and culture conditions. In all experiments, *C. glutamicum* ATCC 13032 (wild type) was used. Cells were grown in Erlenmeyer flasks on a rotary shaker (150 rpm) at 30°C. The following basal salt medium was used (per liter): $(NH_4)_2SO_4$, 5g; urea, 5 g; KH_2PO_4, 2 g; $K_2HPO_4 \cdot 3H_2O$, 2 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $MnSO_4 \cdot H_2O$, 0.01 g; $CaCl_2 \cdot 2H_2O$, 0.01 g; $ZnSO_4 \cdot 7H_2O$, 0.03 mg; H_3BO_3, 0.1 mg; $CoCl_2$, 0.07 mg; $CuCl_2 \cdot 2H_2O$, 0.03 mg; $NiCl_2 \cdot 6H_2O$, 0.01 mg; $NaMoO_4 \cdot 2H_2O$, 0.1 mg; glucose, 50 g; biotin, 1 μg (biotin limited) or 200 μg (biotin supplemented); pH 7.0. For washing procedures, glucose was omitted from this medium. Cell mass was determined by measuring the optical density at 600 nm. An optical density of 10 at 600 nm corresponds to a cell mass of 2.1 mg (dry weight)/ml.

Short-term fermentation. For all experiments, we used short-term fermentation, which has proved to be well suited for investigations concerning glutamate secretion. The benefits of this system for amino acid efflux experiments have been discussed earlier (14). For this procedure, cells were harvested in the early production phase. To obtain maximal and reproducible secretion rates, the bacteria were treated under defined conditions, including several washing and preincubation procedures. Finally, the cells were suspended in basal salt medium lacking biotin for starting short-term fermentation (14).

Chemicals. [^{14}C]taurine, [U-^{14}C]acetate, and ^3H_2O were purchased from Amersham International, Buckinghamshire, United Kingdom. Biochemicals were obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany or Sigma Chemical Co., St. Louis, Mo. All other chemicals were of analytical grade.

Extraction of intracellular amino acids. Cells were separated from the medium by silicone oil centrifugation, using silicone oil (PN 200; Bayer) as the separation layer and 20% $HClO_4$ as the acid bottom layer, by the method of Klingenberg and Pfaff (24). The sedimented cells in the perchloric acid layer were extracted by sonication, and the extracts were neutralized by adding KOH-triethanolamine (14). After cooling on ice, the extracts were centrifuged and the supernatants were used for amino acid determination. Glutamate was determined enzymatically by the method of Beutler and Michal (1).

Determination of intra- and extracellular volume and intracellular glutamate concentration. The intra- and extracellular water volumes of the cells were determined by incubation with ^3H_2O and [^{14}C]taurine and subsequent separation from the medium by silicone oil centrifugation (34). For calculation of the intracellular amino acid concentration, the corrected amounts of internal amino acids were divided by the intracellular water volumes of the cells.

Determination of total phopholipid content: extraction method 1. Aliquots of the fermentations (optical density at 600 nm, 10 to 20) were stopped by rapid cooling on ice and by subsequent addition of $HClO_4$ (final concentration, 1%). The cells were washed twice in H_2O and extracted by the method of Bligh and Dyer (3) by using sonication for 1 h. The resulting chloroform layers were evaporated to dryness by a stream of argon. Total lipid phosphorus was determined after combustion of the evaporate, using the method of Chen et al. (5).

Determination of fatty acids. Cell suspensions were extracted as described above. For quantification of the fatty acids, a defined amount of phosphatidylcholine-diheptade-

canoyl was coextracted; heptadecanoic acid (17:0), which does not occur in *C. glutamicum*, was used for internal standardization. The evaporates of the chloroform layers were transesterified with 2 N HCl in methanol for 15 h at 85°C. After triple extraction of the methyl esters with *n*-hexane, the collected hexane layers were evaporated to dryness by a stream of argon and dissolved in 300 μl of *n*-hexane. Fatty acid esters were analyzed by gas chromatography in a Hewlett-Packard 5890A gas chromatograph equipped with a capillary column.

Incorporation of [^{14}C]acetate into total lipid: extraction method 2. [^{14}C]acetate (57 mCi/mmol) was added to cell suspensions (0.5 μCi/ml) of short-term fermentations (optical density at 600 nm, 10 to 20). After appropriate time intervals, 200-μl aliquots of the reaction mixture were added to 100 μl of ice-cold 20% $HClO_4$. The mixture was centrifuged and the resulting pellet was washed twice with 200 μl of 5% $HClO_4$. After triple extraction with 150 μl of chloroform-methanol (2:1, vol/vol), the collected supernatants were washed twice with equal volumes of H_2O. The organic layer was evaporated to dryness and dissolved in chloroform-methanol (2:1, vol/vol). Aliquots were counted in a liquid scintillation counter.

Separation of phospholipids. Cell suspensions were extracted as described above (extraction method 1), and the resulting evaporates were suspended in chloroform-methanol (1:2, vol/vol). Aliquots were separated by two-dimensional thin-layer chromatography (2D-TLC), using silica gel 60 TLC plates (Merck AG, Darmstadt, Federal Republic of Germany). The solvent for the first dimension was chloroform-methanol-ammonia, 65:25:5 (vol/vol/vol); that for the second dimension was chloroform-methanol-acetone-acetic acid-H_2O, 30:10:40:10:5 (vol/vol). Phosphatidic acid (PA), phosphatidylinositol (PI), diphosphatidylglycerol (DPG) and phosphatidylglyerol (PG) were identified by 2D-TLC, comparing them with reference substances. Since PI mannoside (PIM) and PI could not be differentiated by specific reagents (see below), PIM was identified by its R_f value after separation by one-dimensional TLC (27, 48). The following solvent system was used: diisobutyl ketone-acetic acid-H_2O, 40:25:5 (vol/vol/vol). The PIM spot identified on one-dimensional TLC was assigned to the corresponding phospholipid spot of 2D-TLC.

Lipids separated on TLC plates were detected by the following staining procedures (16): (i) total lipid with J_2 vapor; (ii) sugar-containing lipids (PI, PIM, no phosphorus-containing glycolipids) with periodate-Schiff stain; (iii) acidic phospholipids (PA, PI, PIM, PG, DPG, X) with rhodamine 6G stain; (iv) amino nitrogen-containing lipids with ninhydrin stain; and (v) phosphorus-containing lipids with ammonium molybdate stain. A 2D-TLC of lipids of *C. glutamicum* is shown in Fig. 1.

Quantitative analysis of single phospholipids by estimation of phosphorus and incorporated [^{14}C] acetate. The phospholipids were scraped off the 2D-TLC plates, eluted with chloroform-methanol (1:2, vol/vol), and evaporated. Phospholipid amounts were determined by phosphorus analysis by the method of Chen et al. (5) after combustion of the evaporates. For rapid analysis of the time course of the increase of single phospholipids after biotin addition, synthesis of single phospholipids was followed by measurement of [^{14}C]acetate incorporation, since a slight increase of minor phopholipid components within short time intervals cannot be detected chemically. [^{14}C]acetate (57 mCi/mmol) was added to cell suspensions of short-term fermentations (0.5 μCi/ml) 5 min before biotin addition. After short time
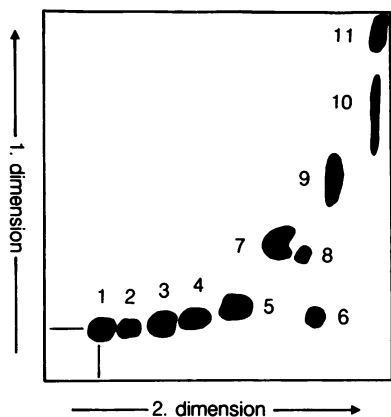
FIG. 1. Two-dimensional TLC of lipid extracts of biotin-limited, glutamate-producing *C. glutamicum*: first dimension: chloroform-methanol-ammonia, 65:25:5 (vol/vol/vol); second dimension: chloroform-methanol-acetone-acetic acid-H_2O, 30:10:40:10:5 (vol/vol). 1, Origin; 2, unknown lipid containing only trace amounts of phosphorus; 3, PI; 4, PIM; 5, X; 6, PA; 7, PG; 8, DPG; 9, glycolipid; 10, free fatty acids; 11, less polar lipids.

intervals, aliquots were extracted (method 1) and the phospholipids were separated by 2D-TLC. Spots of phospholipids were scraped off and counted in a liquid scintillation counter. To correlate the incorporated radioactivity with the amounts of newly synthesized phospholipids, the single phospholipids were chemically analyzed after relatively long time intervals (compared with the detection of [14C]acetate incorporation), sufficient to guarantee a significant increase of lipid amounts. Chemical analyses were carried out immediately before and 18 min after biotin addition. The chemically determined amounts of newly synthesized phospolipids were then correlated with the incorporated radioactivity, which was measured at identical times. Based on the obtained correlations, which may be different for every single phospholipid, and assuming that the specific radioactivities of incorporated [14C]acetate in every newly synthesized phospholipid are identical, the incorporated radioactivity, measured after short time intervals, could then be converted into lipid amounts for individual phospholipids.

RESULTS

The following experiments with *C. glutamicum* under biotin limitation were carried out to elucidate whether there exists a functional correlation between changes in the physical state of the membrane and the ability to secrete glutamate. Thus, we had to analyze changes in the lipid content and composition, on the one hand, and changes in kinetic and energetic properties of substrate transport across the membrane, on the other hand.

Lipid state of glutamate-secreting cells. First, the total content of membrane lipids in *C. glutamicum* under our experimental conditions of controlled fermentation was determined, represented by the total fatty acids and phospholipids. Furthermore, the distribution of individual fatty acids was analyzed by gas chromatography and the different classes of phospholipids were separated by TLC and estimated chemically.

Table 1 gives a complete overview of these parameters. As expected, there is a substantial difference between producer and nonproducer cells. The total amount of lipids, represented by the fatty acids, as well as the phospholipid content

TABLE 1. Phospholipids and fatty acids of *C. glutamicum*[a]

Cells	Fatty acids		Total phospholipids (nmol/mg dry wt)	% of total fatty acids or total phospholipids										
	μg/mg, dry wt	Saturated/unsaturated		Myristic acid (14:0)	Palmitic acid (16:0)	Palmitoleic acid (16:1)	Stearic acid (18:0)	Oleic acid (18:1)	PI	PIM	PG	DPG	PA	X
Producer	10.8 ± 0.5	1.11 ± 0.05	17 ± 1.1	2.3 ± 0.3	47.8 ± 1.1	1.8 ± 0.1	1.3 ± 0.2	46.5 ± 1.5	6.6 ± 0.6	7.7 ± 0.7	69.5 ± 3.1	4.2 ± 0.5	5.4 ± 0.6	6.5 ± 0.7
Nonproducer	20 ± 0.8	0.71 ± 0.05	32 ± 1.5	0.3 ± 0.1	40.2 ± 1.2	0.4 ± 0.1	1.2 ± 0.1	57.9 ± 1.5	3.2 ± 0.04	4.9 ± 0.5	87 ± 1.9	1.0 ± 0.4	2.5 ± 0.4	1.4 ± 0.3

[a] Lipid content and composition were compared in dependence on the state of glutamate secretion. Producer cells were grown under biotin limitation, whereas nonproducer cells are biotin-supplemented cells. Data are average values of at least three determinations.

are decreased by about 50%; also, the ratio of saturated/ unsaturated fatty acids is definitely changed in biotin-limited cells. These basic data are in agreement with published results (15, 37, 38, 45). The analysis of individual fatty acids revealed both a relative increase in palmitic acid and a relative decrease in oleic acid, which were responsible for the change in the ratio of saturated/unsaturated fatty acids. In the analysis of fatty acids of total lipid from *C. glutamicum* by gas chromatography, we could not detect any further component in substantial amounts besides those reported in Table 1.

For complete phospholipid analysis, the bacterial membranes were extracted, and the extracts were separated by 2D-TLC. The individual phospholipids were identified by specific staining procedures and quantitated by phosphate analysis. One component, named X, could not be identified; corresponding color reactions indicated an acidic phopholipid similar to PA, and its identity will be discussed later. We could not detect any nitrogen-containing phospholipid. Although the total content of phopholipids was dramatically decreased, the distribution of the individual species was not extensively changed (Table 1). The relative amount of the major component, PG, was somewhat decreased in producer cells corresponding to a relative increase in all of the other minor components.

Correlation of membrane changes with glutamate secretion. For direct comparison of *C. glutamicum* cells under secreting and nonsecreting conditions, bacterial cells in a short-term fermentation under the condition of active glutamate production were separated into two portions, and biotin was added to one of the aliquots. In the two different populations, i.e., biotin-limited cells and cells with added biotin, the kinetics and energetics of glutamate transport as well as the changes in the membrane state were determined in short time intervals after addition of biotin. Thus, it should be possible to elucidate whether changes in the two different aspects (membrane state and secretion behavior) in fact happened in an identical time scale and could be assumed to be correlated functionally.

Addition of biotin immediately decreased the efflux of glutamate to a level of about 20% of that of untreated producer cells (Fig. 2A). After biotin addition, the concentration of internal glutamate did not change and the cells started to grow (not shown). The general effect of biotin addition on lipid synthesis was tested by using incorporation of [^{14}C]acetate as a monitor of fatty acid synthesis (Fig. 2B). Only trace amounts of label were incorporated into biotin-limited producer cells, whereas immediate and strong labeling of the total lipid by [^{14}C]acetate could be observed after addition of biotin. However, incorporation of [^{14}C]acetate could not be directly quantitated since the main part of acetyl-coenzyme A used for lipid synthesis comes from the substrate glucose. Therefore, we analyzed the effect of biotin addition on the total content of fatty acids. Figure 2C clearly shows that biotin caused an increase of total lipid. The original lipid/protein ratio of biotin-supplemented cells, however, was reached only after 2 h. It is obvious that the amount of newly synthesized fatty acids within the first minutes, i.e., within the time range in which glutamate secretion is already maximally decreased, is negligible, <5% of the original lipid content.

Since the altered ratio of saturated/unsaturated fatty acids also had been suggested to be the reason for glutamate secretion under biotin limitation, we investigated the immediate effects of biotin addition on this parameter (Fig. 2D). As expected, after 10 to 20 min, biotin-limited cells changed
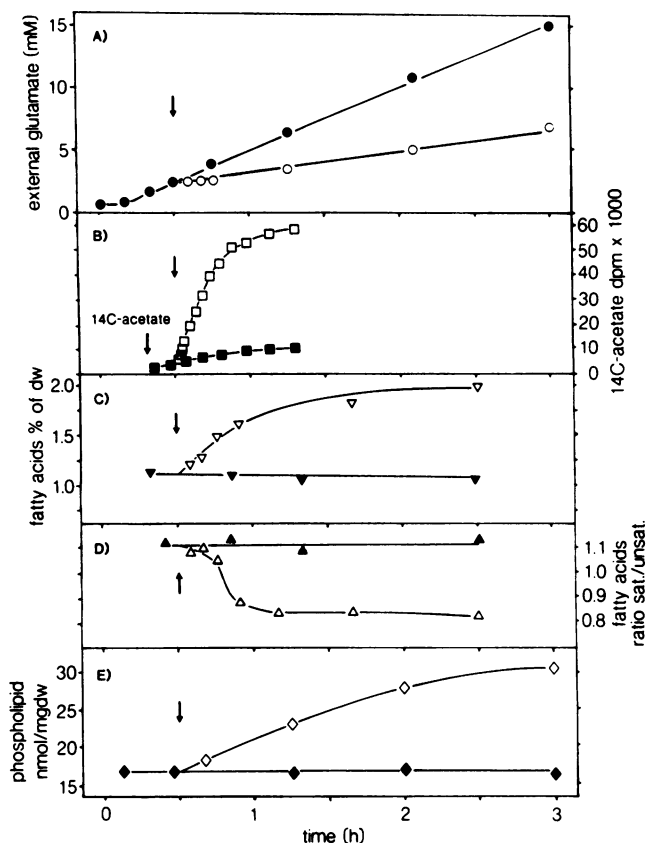

FIG. 2. Short-term fermentation of *C. glutamicum*. Effect of biotin addition on biotin-limited producer cells. Untreated (closed symbols) and biotin-treated (open symbols) cells were compared. Arrows symbolize biotin addition. (A) Secretion of glutamate. (B) Activity of fatty acid synthesis indicated by incorporation of [^{14}C] acetate into total lipid. [^{14}C]acetate was added when indicated. (C) Fatty acid content. (D) Ratio of saturated/unsaturated fatty acids. (E) Phospholipid content. dw, Dry weight.

the ratio of fatty acid saturation towards a higher content of unsaturated fatty acids; however, as in the case of fatty acid synthesis, the time scale of this effect in no way correlated with the immediate stop of glutamate secretion. Also, during the early phase of fatty acid synthesis after biotin addition we did not detect any unexpected or specific fatty acid to be significantly increased.

Similar to fatty acids, the content of phospholipids increased to values obtained for biotin-supplemented cells, but only more than 2 h after biotin addition (Fig. 2E). Similar to all other parameters described above, the increase within the first minutes was <5%.

Since neither of these parameters could be related to the rapid shift from effective glutamate production to low-level secretion, another obvious explanation had to be tested. It could be possible that a specific, perhaps regulatory lipid component, synthesized in small amounts and thus not detectable in the total lipid analyses besides the surplus of other components, was responsible for the switch in secretion behavior. Since a specific fatty acid could not be observed (see above), we thoroughly analyzed the time course of the appearance of newly synthesized phopholipid species after biotin addition to detect a possible regulatory component. The phospholipids synthesized after biotin addition were labeled with [^{14}C]acetate and separated by
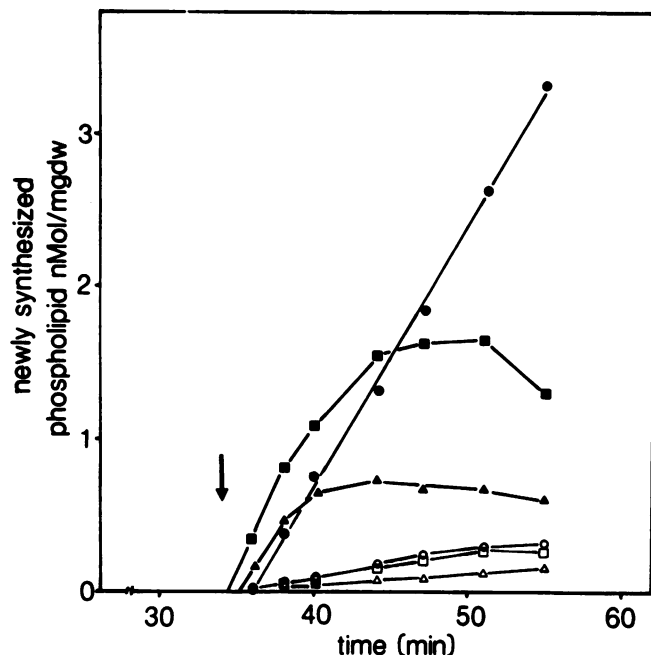
FIG. 3. Effect of biotin addition on synthesis of single phospholipids of biotin-limited nonproducer cells during a short-term fermentation. The amounts of newly synthesized PG (●), PA (■), X (▲), PI (□), PIM (△), and DPG (○) were determined. Arrow indicates biotin addition. dw, Dry weight.
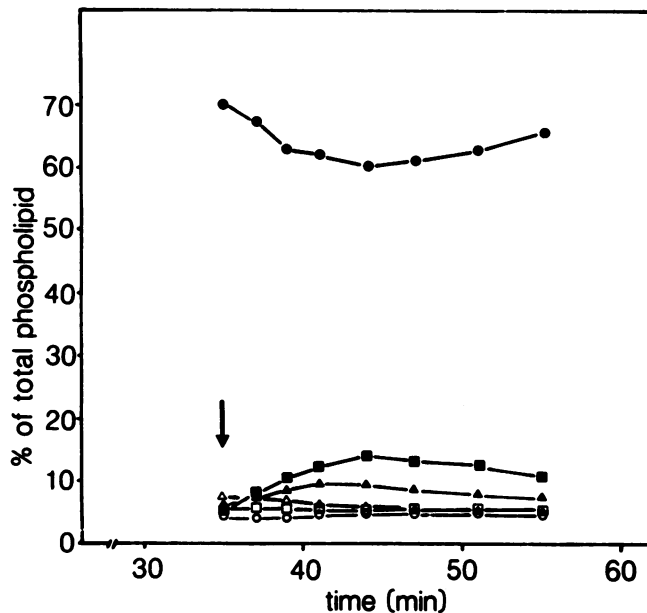
FIG. 4. Effect of biotin addition on phospholipid composition of biotin-limited producer cells during a short-term fermentation. The relative amounts of single phospholipids were determined for PG (●), PA (■), X (▲), PI (□), PIM (△), and DPG (○). Arrow indicates biotin addition. The phospholipid composition of untreated cells remained constant on the level at the time of biotin addition (shown in Table 1.)

2D-TLC. The quantitative determination was carried out by phosphate analysis of every single phospholipid at representative times (see Materials and Methods). Thereby, we obtained the specific radioactivity of every single newly synthesized phospholipid; the time scale of synthesis could thus be followed (Fig. 3). Within this time range, the amounts of newly synthesized phospholipids without biotin addition were below the detection limit, i.e., <3 to 4% of that of biotin-treated cells. Biotin addition caused an instant beginning of the synthesis of all kinds of phospholipids, that of PA appearing somewhat earlier than the others. The rates of synthesis of the individual phospholipids more or less correlated with their relative amounts in the membrane. The synthesis of a new, so far undetected component could not be observed. By combining the data of phospholipid labeling (Fig. 3) and phospholipid composition (Table 1), the relative content of every single phospholipid could be calculated. Thus, the relative distribution of the individual phospholipids within the time course of the inhibition of glutamate secretion after biotin addition could be analyzed in detail (Fig. 4). The relative amount of PG decreased in the period of active lipid synthesis, although it was also synthesized in that period of time, whereas the content of PA and, somewhat later, also that of the acidic phospholipid X increased. These small changes mainly in PA and X will be explained in detail in the Discussion. Besides these unspecific effects, no significant changes in phospholipid distribution could be detected.

Fatty acid synthesis is the target of biotin addition. When investigating the consequences of biotin limitation, there is another important question. As mentioned in the introduction, direct effects of biotin on enzymes besides acetyl-coenzyme A carboxylase (25) and possibly on carrier proteins (10) have to be taken into account. We approached this question again by investigating the effect of biotin during short-term fermentation; however, we also applied cerulenin, which is reported to be a specific inhibitor of the condensing enzyme (β-ketoacyl-ACP synthase) of the fatty acid synthase complex (13, 33). The inhibitory influence of cerulenin on the isolated fatty acid synthase has also been shown in *B. ammoniagenes* (17), which is closely related to *C. glutamicum* (42). Addition of 200 μg of cerulenin per ml instantly stopped incorporation of [^{14}C]acetate into newly synthesized fatty acids in growing biotin-supplemented cells of *C. glutamicum* (Fig. 5). This inhibitor is thus a well-suited compound to study the importance of the activity of fatty acid synthase in the present experiments.

Cerulenin was able to block the influence of biotin on glutamate-producing cells completely (Fig. 6). Again, as already shown above, addition of biotin led to an instant and drastic decrease in glutamate secretion. When cerulenin was added together with biotin, the inhibition of glutamate efflux by biotin was completely abolished; i.e., simultaneous addition of biotin and cerulenin had virtually no effect on actively secreting bacteria. Cerulenin, even when added 6 min after biotin, was able to revert the effect of biotin completely within a few minutes; i.e., the secretion rate was restored to the original value as compared with untreated producer cells. In other words, in these experiments cerulenin phenomenologically, although not functionally, counteracted the influence of biotin on the level of fatty acid synthesis.

Consequently, addition of cerulenin alone should mimic the withdrawal of biotin from *C. glutamicum*. This experiment is described in Fig. 7A, indicating that, after addition of cerulenin to biotin-supplemented growing cells, the bacteria started to secrete glutamate. Within 2 h, a high efflux rate of about 12 μmol/min per g (dry weight) was achieved, comparable to that obtained in normal short-term fermentations (14). To compare cerulenin-induced efflux with the detailed
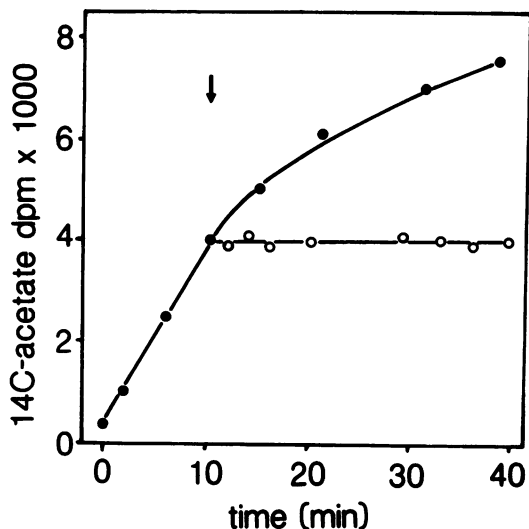
FIG. 5. Influence of cerulenin on fatty acid synthesis. Fatty acid synthesis is indicated by incorporation of [^{14}C]acetate into total lipid of biotin-supplemented, growing producer cells during a short-term fermentation. Symbols: ●, untreated cells; ○, effect of cerulenin addition (indicated by arrow).

lipid analysis of secreting and nonsecreting cells as described above, we further measured the phospholipid content of *C. glutamicum* in the course of these experiments (Fig. 7B). The amount of phospholipids decreased within 2 to 3 h after cerulenin addition to about 50% of biotin-supplemented cells. This value was identical to that obtained for biotin-limited producer cells. Only when the phospholipid content of the bacterial cells was reduced to values lower than about 70% of the untreated cells (Fig. 7B) could effective glutamate secretion be observed.

DISCUSSION

In the literature, in general, changes in the lipid membrane of coryneform bacteria were held responsible for effective
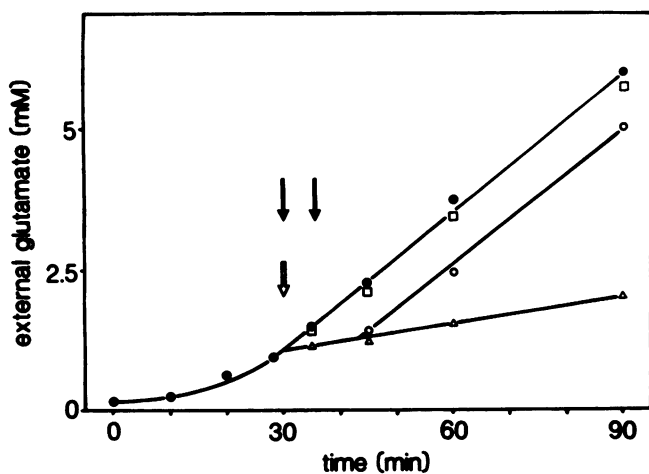

FIG. 6. Short-term fermentation of *C. glutamicum*: effect of cerulenin on inhibition of glutamate secretion by biotin addition. Glutamate secretion of untreated cells (closed symbols) and that of biotin-treated cells (open symbols) were determined. Open arrow indicates addition of biotin. Open triangles represent external glutamate of cells treated with biotin only. Cerulenin (↓) was added together with (□) or 6 min after (○) biotin.
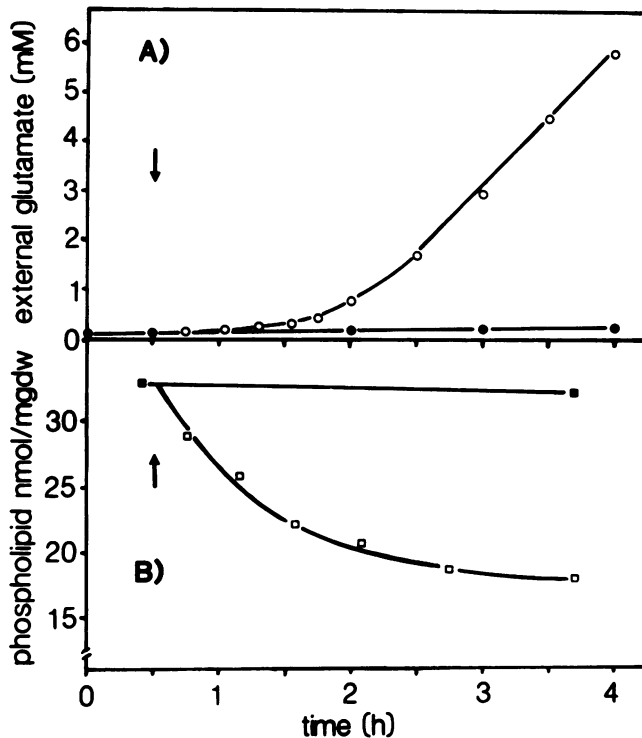

FIG. 7. Short-term fermentation with biotin-supplemented non-producer cells of *C. glutamicum*: effect of cerulenin addition. Untreated (closed symbols) and cerulenin-treated (open symbols) cells were compared. Arrow indicates cerulenin addition. (A) Glutamate secretion. (B) Phospholipid content. dw, Dry weight.

glutamate secretion in biotin-limited cells (4, 9, 21, 22, 30). Since we showed recently that glutamate efflux cannot be explained by passive diffusion through the altered membrane but on the contrary is mediated by a specific excretion carrier system (14), it was of basic interest to investigate whether there is, in fact, a close correlation between the lipid composition of the plasma membrane and the activity of glutamate secretion and whether the altered membrane is the ultimate reason for the ability to secrete glutamate. It was important for our studies that we use a new experimental approach by which the same cells could be forced to change directly between producing and nonproducing states, in contrast to experiments published so far in which two completely different cell populations, i.e., biotin-supplemented nonproducer and biotin-limited producer cells, were always compared.

When we first applied the conventional approach, i.e. analysis of the two different cell populations (Table 1), biotin supplemented and biotin limited, we could confirm the previously published results to a large extent (8, 37, 38, 43, 45). Both the lipid content and the composition of producer cells were altered.

The alternative new experimental approach, which proved to be essential for elucidating the relation of the membrane state and efflux behavior, was a detailed lipid analysis within the first minutes after addition of biotin to glutamate-secreting cells and a direct correlation with amino acid efflux activity. Our experimental results provide strong evidence against a direct relation of the lipid state and efflux activity (Fig. 2A to E). Biotin addition caused an instant decrease of glutamate secretion, whereas neither the amount of newly synthesized fatty acids nor the phospholipid content

changed significantly within the time range in which the full effect on glutamate secretion could already be observed. Also, the ratio of saturated/unsaturated fatty acids was changed in a much slower time scale. The important energetic parameters, i.e., chemical potential of glutamate and internal glutamate concentration, remained unchanged after biotin addition (not shown). These results demonstrate that effective glutamate efflux is not an obligatory consequence of the altered lipid composition of the membrane.

An alternative hypothesis, however, had to be taken into consideration. Several authors suggested that glutamate secretion may be induced by the presence of a specific phospholipid component (20, 45). Both PIM and the amount of PA and DPG have been made responsible for regulating amino acid efflux (26, 37). Thus, we analyzed the time course of the relative amount of every single phospholipid immediately after addition of biotin to biotin-limited producer cells. The only significant changes we observed were a relative increase in PA and an undefined component, X. Concomitantly with the transient increase of several of the minor phospholipid components, a transient decrease of the major component, PG, is easy to rationalize. The transient increase of PA was by no means unexpected since PA is the precursor of the other phopholipids. Thus, the time course of PA concentration in the cells after biotin addition and onset of lipid synthesis closely followed the pattern which would have been expected for a precursor molecule. Also, component X, which is an acidic phospholipid and lacks sugar and nitrogen components, closely followed the "precursorlike" time course of PA. It is thus reasonable to assume that X is another precursor molecule of the major phospholipid PG, namely, CDP-diacylglycerol or PG-phosphate (2, 12).

Besides the lack of correlation between the decrease in glutamate secretion and significant changes in the lipid composition, we obtained an even stronger argument against a direct functional relation by the experiments described in Fig. 6. If the subtle changes in the lipid content within the first minute(s) after biotin addition were responsible for the instant decrease in glutamate secretion, this effect, hypothetically due to an altered membrane, should on no account be reverted immediately on blocking fatty acid synthesis again by cerulenin.

The conclusion that the changed lipid composition cannot be the direct trigger for glutamate secretion is further corroborated by a comparison of different bacteria. The phospholipid composition as determined here is more or less in agreement with data published for *C. glutamicum* (15) and *B. ammoniagenes* (48). It differs significantly, however, from other coryneform glutamic acid-producing organisms which show extensive variations in the major components and in the occurrence of nitrogen-containing phospholipids (20, 26, 37).

The arguments put forward so far demonstrate that efflux is not causally related to lipid composition. However, there remained two important questions with respect to the importance of the lipid state. (i) Is a decrease in lipid content of the membrane, although not the only factor, in principle a necessary requirement for glutamate secretion? (ii) Are the effects of biotin deficiency, on the one hand, and biotin addition, on the other hand, in fact directly related to the activity of fatty acid synthesis or possibly (also) to some other biotin-dependent enzyme systems in the cell?.

By using cerulenin, a specific inhibitor of fatty acid synthase (Fig. 5 and 6), we could show that cerulenin directly counteracted the effect of biotin on both fatty acid synthesis and glutamate secretion. It was possible to switch from high- to low-level secretion and back again within a time scale of minutes or less. We conclude that the influence of biotin is directly related to its effect on fatty acid synthesis, possibly by a change in metabolic consequences related to fatty acid synthesis, although we proved (see above) that the lipid state of the membrane is not the only parameter for inducing glutamate efflux. Other possible explanations of the influence of biotin could be ruled out, i.e., an effect via other biotin-dependent enzymes or carriers (10, 25).

Consequently, it was interesting to test the direct effect of cerulenin on the activity of glutamate efflux. As could have been expected from its inhibiting effect on lipid synthesis, cerulenin in fact led to effective secretion of glutamate. However, this induction took place only after the lipid content of the cell has been significantly reduced.

We therefore conclude that, in addition to the obviously essential prerequisite for effective glutamate secretion, i.e., reduced lipid content of the membrane, another regulating factor(s) is necessary. This was shown by the detailed analysis of the membrane state and efflux behavior immediately after biotin addition to producer cells. We have evidence that there is a superior hierarchy of regulating events which, in addition to membrane changes, are essential for effective glutamate secretion; the respective effectors are parameters of the energy state of the bacterial cell. These regulation phenomena related to glutamate secretion are under investigation.

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