Amino Acid Biosynthesis – Pathways, Regulation and Metabolic Engineering

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Production of Glutamate and Glutamate-Related Amino Acids: Molecular Mechanism Analysis and Metabolic Engineering

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Abstract Glutamate production is a typical success in industrial fermentation. Annual production of glutamate by *Corynebacterium glutamicum* is over 1.5 million tons per year worldwide. It is well known that there are some triggers of glutamate overproduction by *C. glutamicum*: depletion of biotin, which is required for cell growth; addition of detergent; addition of β-lactam antibiotics such as penicillin; and addition of ethambutol or cerulenin. A marked change in metabolic pathways occurs after glutamate overproduction is triggered. In this chapter, recent studies on the molecular mechanisms of glutamate production are described with a particular focus on triggering mechanisms, changes in key enzyme activities, and secretion of glutamate. Recent advances in genome-wide studies, including genomics, proteomics, metabolomics, and on metabolic flux analysis of flux redistribution during glutamate overproduction are discussed as well. The biosynthesis of the related amino acids glutamine and proline and strategies for their overproduction are also described.

Keywords Glutamate · Glutamine · Proline · Metabolic engineering · Triggering of glutamate overproduction

1 Glutamate

In this section, recent studies on the molecular mechanisms of glutamate production and metabolic engineering analysis of flux redistribution in the central and anaplerotic pathways are described.

1.1 General Introduction and History

Monosodium glutamate is a substance that exhibits the specific taste of "Umami", well-known in Japanese cuisine. It was discovered and isolated from hydrolyzates of "konbu", seaweed, in 1908 by Dr. Kikunae Ikeda. In the presentation at the 8th International Congress of Applied Chemistry, Chicago, 1912 he reported that: "*An attentive taster will find out something common in the complicated taste of asparagus, tomatoes, cheese and meat, which is quite peculiar and cannot be classified under any of the well defined four taste qualities, sweet, sour, salty and bitter*". He succeeded in identification of this substance as monosodium glutamate. Ajinomoto was the first company to produce monosodium glutamate on an industrial scale by extraction from wheat hydrolyzates. A coryneform bacterium, *Corynebacterium glutamicum* (former name *Micrococcus glutamicus*), was isolated from a soil sample in

the 1950s by Japanese researchers of Kyowa Hakko Kogyo (Kinoshita et al. 1957; Udaka 1960). They developed a fermentation method to produce glutamate directly from cheap sugar and ammonia, thus reducing production costs of glutamate considerably. Currently, *C. glutamicum* is widely used as a producer of amino acids such as lysine, arginine, histidine, valine, and so forth (Eggeling and Sahm 1999, Kimura 2003, Ikeda 2003).

The market of amino acids is increasing with an annual growth rate of 5–7% (Leuchtenberger et al. 2005). With the exploitation of the wide spectrum of the uses of amino acids as food additives, pharmaceuticals, feed supplements, cosmetics, and polymer precursors, the demand for amino acids has increased. The biggest market among the amino acids is that of glutamate, and the main use of this amino acid is as a flavor enhancer. Recently, the annual production of glutamate was more than 1.5 million tons per year worldwide (Ajinomoto 2006).

C. glutamicum is a facultatively anaerobic, non-spore-forming, Gram-positive bacterium. This microorganism has the ability to produce large amounts of amino acids such as glutamate and lysine. Recently, the genome sequencing of *C. glutamicum* was completed (Ikeda and Nakagawa 2003, Kalinowski et al. 2003). A related species, *Corynebacterium efficiens*, was found to be another glutamate producer (Fudou et al. 2002) and the genome sequencing of this strain was also performed (Nishio et al. 2003).

There are some triggers of glutamate overproduction: depletion of biotin, which is required for cell growth: addition of detergent; addition of β-lactam antibiotics such as penicillin; addition of ethambutol or cerulenin; and for temperature-sensitive mutants a temperature shock. A marked change in metabolic flux typically occurred after these triggers.

1.2 Brief Summary of Glutamate Production by *C. glutamicum*

Glutamate is synthesized from 2-oxogluratrate by a one-step reaction catalyzed by glutamate dehydrogenase (GDH) (Börmann et al. 1992), which is the main pathway for glutamate formation when the ammonium concentration is sufficiently high (Ertan 1992a,b; Börmann-El Kholy et al. 1993). 2-Oxoglutarate is a member of the tricarboxylic acid cycle and, thus, of the central carbon metabolism, including the glycolytic pathway, the pentose phosphate pathway, and the anaplerotic pathways, which should be discussed for overproduction of glutamate.

C. glutamicum requires biotin for cell growth. Glutamate is not produced by wild-type *C. glutamicum* when excess biotin is present in the culture medium. However, a significant production of glutamate occurs when biotin is depleted (Shiio et al. 1962). Even with excess biotin, the addition of a detergent compound such as polyoxyethylene sorbitan monopalmitate (Tween 40) or polyoxyethylene sorbitan monostearate (Tween 60) causes *C. glutamicum*

to produce significant glutamate (Takinami et al. 1965). Addition of penicillin, one of the β-lactam antibiotics, also enhances the overproduction of glutamate by *C. glutamicum* (Nara et al. 1964). Similarly, the addition of ethambutol (Radmacher et al. 2005), a chemotherapeutic used to treat tuberculosis, or the addition of cerulenin (Hashimoto et al. 2006) trigger glutamate production by wild-type *C. glutamicum*. Temperature-sensitive mutants produce glutamate after a heat-shock (Momose and Takagi, 1978; Delaunay et al. 1999, 2002), although this process is not stable (Uy et al. 2003).

Since biotin is a cofactor of acetyl-CoA carboxylase, which is necessary for fatty acid synthesis, it was thought that the cell membrane permeability increased when biotin was depleted in the culture medium. Similarly, addition of detergent, penicillin, ethambutol, or cerulenin alters the composition of the cell membrane or cell wall of this microorganism. Consequently, it was thought that the permeability of the cell membrane and cell wall should change due to these operations. This explanation was called the "leak model" of glutamate production. However, from the viewpoint of the material balance of intracellular and extracellular glutamate, it is difficult to explain the high accumulation of glutamate to more than 60–80 g/L by this model alone.

Very early on, it was proposed that *C. glutamicum* produces glutamate because it might lack 2-oxoglutarate dehydrogenase complex (ODHC) and all flux from isocitrate to 2-oxoglutarate is channeled to glutamate. However, about 40 years ago *C. glutamicum* was shown to possess ODHC activity (Shingu and Terui 1971; Shiio and Ujigawa-Takeda 1980) and the genes encoding the subunits of ODHC were later characterized (Usuda et al. 1996; Schwinde et al. 2001). The proposal that the activity control of ODHC might be critical for glutamate production (Kinoshita 1985) was substantiated in recent studies (Kawahara et al. 1997) and the signal transduction pathway for regulation of ODHC, which involves protein kinase PknG and the specific inhibitory protein OdhI, were unraveled in 2006 (Niebisch et al. 2006).

1.3 Recent Advances in Analysis of the Mechanism of Glutamate Overproduction by *C. glutamicum*

ODHC is a branch-point enzyme complex between the tricarboxylic acid (TCA) cycle and glutamate biosynthesis and catalyzes the conversion of 2-oxoglutarate to succinyl-CoA. ODHC consists of three subunits, 2-oxoglutarate dehydrogenase (E1o), dihydrolipoamide *S*-succinyltransferase (E2o) and dihydrolipoamide dehydrogenase (E3). The *C. glutamicum odhA* gene encoding the E1o subunit shows high homology with the gene encoding E1o subunit from other bacteria, but the N-terminal extension in *C. glutamicum* OdhA cannot be found in other reported E1os (Usuda et al. 1996).