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Syntheses of glycine and L-serine by their interconversion in the posterior silkgland of the silkworm, *Bombyx mori*

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Summary. It was observed by solution-state ^{13}C NMR spectroscopy that a great portion of the ^{13}C of [1-^{13}C]L-serine fed to the 5th instar larvae of the silkworm, *Bombyx mori* was incorporated into C1 of glycine in silk fibroin. [1-^{13}C]Glycine was detected along with [1-^{13}C]serine in fibroin of the posterior silkgland cultured in a medium containing [1-^{13}C]serine. This formation of [1-^{13}C]glycine was inhibited by addition of aminopterin to the culture medium. These findings suggest that an active conversion from serine to glycine, which needs tetrahydrofolate, occurs in the posterior silkgland for fibroin synthesis. Moreover, the solid-state ^{13}C CP/MAS spectrum of the fibroin prepared from cocoons spun by larvae fed with [^{13}C]formate revealed that serine C3 was labelled specifically with ^{13}C, suggesting that the reverse conversion from glycine to serine took place in the silkworm. The posterior silkgland has the ability to synthesize not only fibroin but also its major materials, glycine and serine.

Keywords: Amino acids – Glycine-serine interconversion – Tetrahydrofolate – Aminopterin – Formate – Fibroin – Silkgland of *Bombyx mori*

Abbreviations: H_4F, tetrahydrofolate; TFA, trifluoroacetate

Introduction

Fibroin is synthesized very actively in the silkworm, *Bombyx mori*. Hence, its four major amino acid components; glycine, alanine, serine and tyrosine, which constitute over 90% of the total amino acid content of fibroin (Kirimura, 1962), are not only derived from amino acids and proteins in the diet, but also synthesized *de novo* in the body (Horie et al., 1978). Organs for these *de novo* syntheses have not yet been determined. Starting materials of the three amino acids except tyrosine are sucrose and glucose in the diet (Steele, 1952; Kasting and McGinnis, 1958). Alanine is synthesized from

pyruvate formed through glycolytic pathway (Garber et al., 1976) and also L-malate in TCA-cycle (Osanai et al., 2000), and serine is from 3-phosphoglycerate, an intermediate of glycolysis (Koide and Shimura, 1961). On the other hand, serine isolated from cocoons spun by *Bombyx* larvae injected with ^{14}C-glycine showed a high radioactivity (Bricteux-Grégoire et al., 1959b; Fukuda, 1960b), suggesting the formation of serine from glycine. On the contrary, serine could be converted directly into glycine (Fukuda, 1960a; Muramatsu et al., 1961). Incorporation of ^{14}C of ^{14}C-formate into glycine in the silk fibroin was less than that into serine (Bricteux-Grégoire et al., 1959a).

Furthermore, glycine must be formed from threonine and also glyoxylate. In fibroin prepared from cocoons spun by larvae injected with radioactive threonine, ^{14}C was incorporated into glycine, alanine and serine with the ratio of 6:1.5:1 (Bricteux-Grégoire et al., 1960). However, there is a little possibility that this essential amino acid would be utilized for syntheses of non-essential amino acids in the body of the silkworm. On the other hand, glyoxylate-glycine aminotransferase activity was high in the posterior silkgland but low in the middle silkgland (Koide et al., 1956; Fukuda and Hayashi, 1958). It was found that glyoxylate derived from glucose (Fukuda and Hayashi, 1960) or citrate (Fukuda and Kameyama, 1961) was present in hemolymph and the silkgland of larva (Fukuda et al., 1954).

Since studies on the formation of glycine from serine and the reverse reaction have been carried out using the whole larval body *in vivo*, it was difficult to analyze this process in detail. In this paper, the interconversion in the posterior silkgland has been clearly confirmed using the *in vitro* culture of this organ alone to avoid interference of metabolic reactions in other organs. Moreover, it is also clarified that tetrahydrofolate (H_4F) and its derivatives participate in these reactions as cofactors, as shown in yeast (Pasternack et al., 1992).

Matrials and methods

Eggs of a cross of the silkworm, *Bombyx mori*, Shuko X Ryuhaku were kindly supplied by Katakura Industry Co., Matsumoto, Japan. These larvae were reared on an artificial diet (Nippon Nosan Industry CO., Yokohama, Japan) (Osanai and Isono, 1996).

For labelling of fibroin with stable isotopes, two techniques, *in vitro* and *in vivo*, were used (Asakura et al., 1991). In the former cases, ^{13}C-formate or [1-^{13}C]L-serine was given to the 5th instar larvae from days 3 to 5 by feeding diet mixed with one isotope. In the later case, fibroin labelled with [1-^{13}C]L-serine was obtained by organ culture of the posterior silkgland in a medium containing the isotope, according to a slightly modified method of Asakura et al. (1993). All solutions, culture media, instruments and tools were sterilized. Procedures were always carried out under sterile conditions. The posterior silkgland was excised from five larvae on day 4 of the 5th instar. After twice washing in 1.15% potassium chloride solution, this organ was cultured for 48 h in a conical glass flask containing fortified Grace's medium with 600 mg/L of [1-^{13}C]L-serine in place of unlabelled L-serine. Then the flask was rotated in an incubator at 25°C under flowing oxygen at 80 rpm with a 25 mm-radius rotation. For culture of the posterior silkgland, in which fibroin is synthesized actively, the original Gace's insect medium (Grace, 1962) was modified to

prepare a fortified Grace's medium, in which concentrations of alanine, tyrosine and glutamate were increased to 2.5, 4.0 and 2.0 times, respectively, and 20 mM of sorbitol-6-phosphate as a stimulant of fibroin synthesis (Asakura et al., 1990) was added with or without 200 mg/L of aminopterin (Sigma Chemical Co., St. Louis, USA) to block the interconversion between glycine and serine.

Two sorts of dried fibroin labelled with [1-^{13}C]L-serine were prepared from the posterior silkgland and cocoon. The posterior silkgland synthesizes and contains only fibroin, while cocoon consists of fibroin and sericin, which is derived from the middle silkgland. 1. The posterior silkgland of larvae cultured with [1-^{13}C]serine was immersed in 30% ethanol solution for 24 h at 4°C to convert fibroin to the β-form. After tissues surrounding the posterior silkgland were removed, the isolated fibroin was dried. 2. Cocoons spun by larvae fed with the same isotope were boiled at 100°C for 30 min in 0.5% Marseille soap solution and washed with distilled water. After this procedure was repeated twice, the degummed fiber was boiled for 1 h in distilled water and dried. Then, each of these dried fibroin preparations was dissolved in 9 M lithium chloride solution to 3%. This fibroin solution was dialysed against distilled water to remove lithium chloride. The solution-state ^{13}C NMR spectrum of the fibroin solution in a 10 mm-tube was observed at 20°C using JEOL α-500 NMR spectrometer (Japan Electron Optic Laboratory) operating at 125.6 MHz. The spectral conditions are as follows: number of free induction decays accumulated, 1,800; spectral width, 33,898 Hz; datapoint, 32,768; 45° pulse angle of duration 15 μsec with 1.0 sec delay between pulses; continuous broad-bond decoupling.

Cocoons spun by larvae fed with [^{13}C]formate were degummed and prepared to the fibroin solution, as described above. After this fibroin solution was dried, the solid-state ^{13}C NMR spectrum of the unaligned fibroin was recorded at 100.4 MHz on a JEOL EX-400 NMR spectrometer equipped with a solid-state ^{13}C-NMR unit operating at 25°C. The spectral conditions are as follows: observed frequency, 100.4 MHz; number of accumulation, 2,000; spectral width, 40.0 kHz; H 90 pulse, 5.0 μsec; pulse delay, 3.0 sec.

Assignments of the amino acid residues of silk fibroin were performed on the basis of the chemical shift data reported previously (Asakura and Murakami, 1985).

Results

Labelling of fibroin with [1-^{13}C]L-serine

The solution-state ^{13}C NMR spectrum of fibroin prepared from cocoons spun by *Bombyx* larvae fed with [1-^{13}C]L-serine showed almost no peak of ^{13}C1 of serine, but one peak of ^{13}C1 of glycine (Fig. 1A), suggesting that active conversion of serine to glycine in this larval body. On the other hand, fibroin synthesized in the posterior silkgland cultured in a medium containing [1-^{13}C]L-serine showed incorporation of original [1-^{13}C]L-serine and also that of *de novo* synthesized glycine (Fig. 1B). This finding suggests that, although folate was removed from this medium, the amount of folate sufficient for the conversion is present in the posterior silkgland. On the contrary, fibroin synthesized in the posterior silkgland which was cultured with the medium containing no folate but its competitive inhibitor, aminopterin, was incorporated primarily with [1-^{13}C]L-serine and a little with [1-^{13}C]glycine (Fig. 1C). This strongly suggests that H_4F and the derivatives are associated with the conversion (Pasternack et al., 1992), which occurs in the posterior silkgland (Fig. 2).

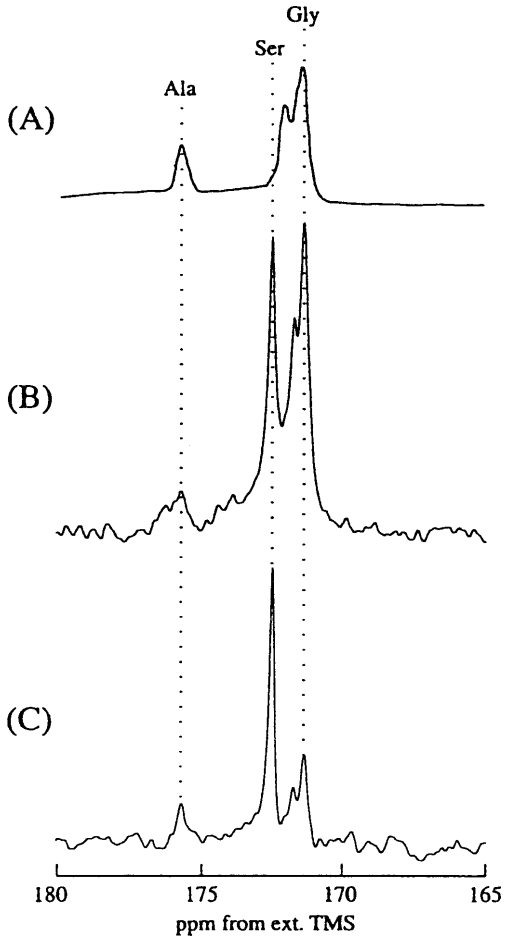

Fig. 1. Solution-state ^{13}C NMR spectra of carbonyl region of silk fibroin of *B. mori*. Labelled fibroins were prepared from either cocoons spun by larvae fed with [1-^{13}C]L-serine (**A**) or silkglands cultured with culture medium containing the same labelled compound with (**C**) and without (**B**) addition of aminopterin

Labelling of fibroin with [1-^{13}C]formate

There is a possibility in the *Bombyx* larva that conversion of glycine to serine may also take place as the reverse reactions of the above formation of glycine from serine, which is related with H_4F derivatives. In these H_4F-related reactions, serine is synthesized by bonding formate to glycine (Fig. 2). The solid-state ^{13}C NMR spectrum of fibroin prepared from cocoons spun by larvae fed with [^{13}C]formate showed specific labelling of ^{13}C to serine C3 (Fig. 3; A and B). Similar results were also obtained from fibroin prepared from cocoons spun by larvae injected with [^{13}C]formate (data not shown).

Discussion

Glycine is produced from serine in the silkworm (Fukuda 1960a; Kawasaki et al., 1968). Since glycine is in great demand as the first major component of

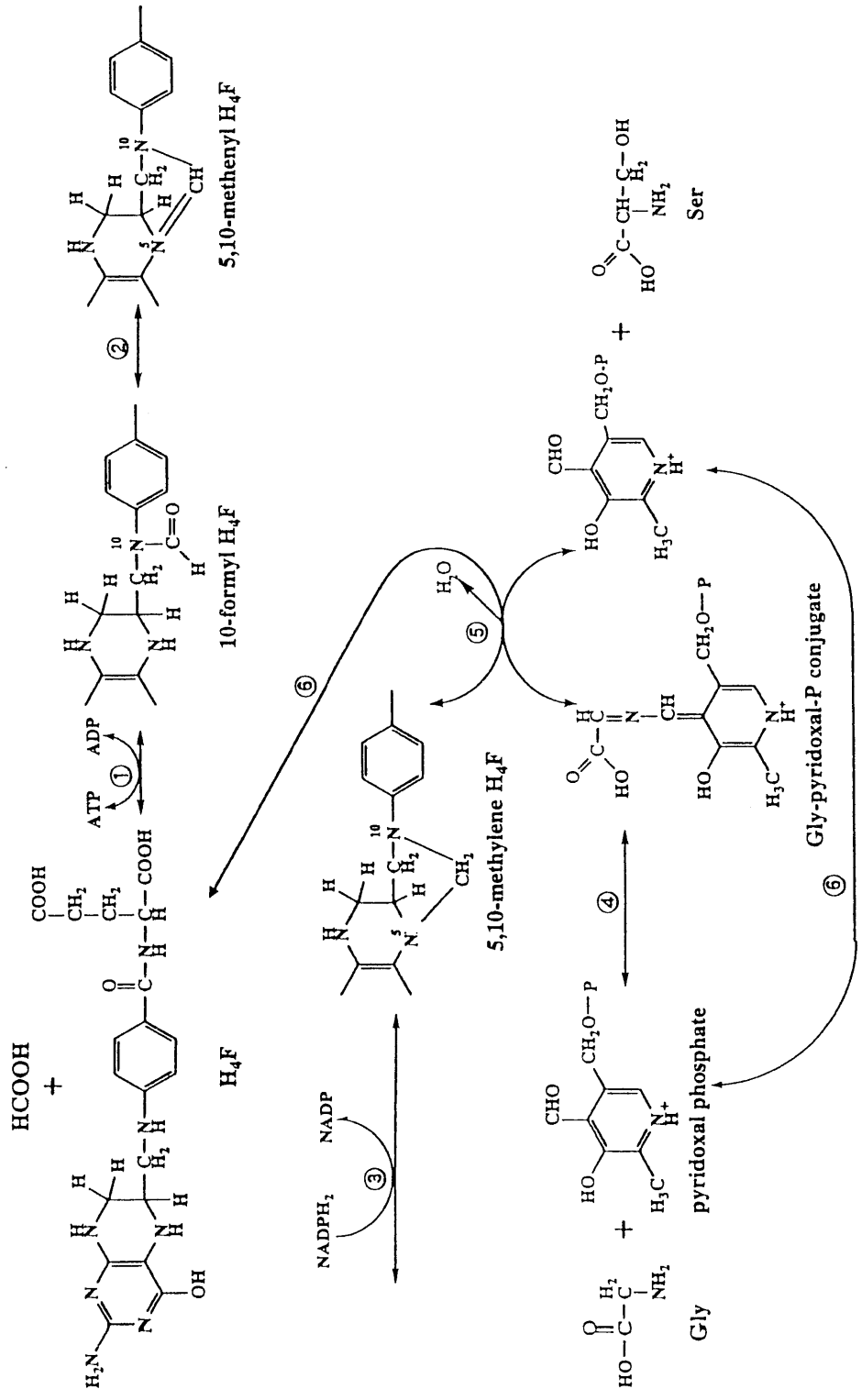

Fig. 2. Formate-mediated interconversion between glycine and serine. *1* Formation of 10-formyl H_4F from formate and H_4F by 10-formyl-H_4F synthase; *2* conversion from 10-formyl-H_4F to 5,10-methenyl-H_4F catalyzed by 5,10-methenyl-H_4F cyclohydrolase; *3* conversion from 5,10-methenyl-H_4F to 5,10-methylene-H_4F catalyzed by 5,10-methylene-H_4F dehydrogenase; *4* formation of a pyridoxal phosphate conjugated with glycine; *5* production of serine, pyridoxal phosphate and H_4F from 5,10-methylene-H_4F and glycine-pyridoxal phosphate conjugate by catalytic action of serine hydroxymethyltransfease; *6* return of H_4F to the reaction 1. and that of pyridoxal phosphate to the reaction 4. Because these reactions, 1.-6., are all reversible, they are involved not only in serine formation from glycine, but also in its reverse reaction. *H_4F* tetrahydrofolate

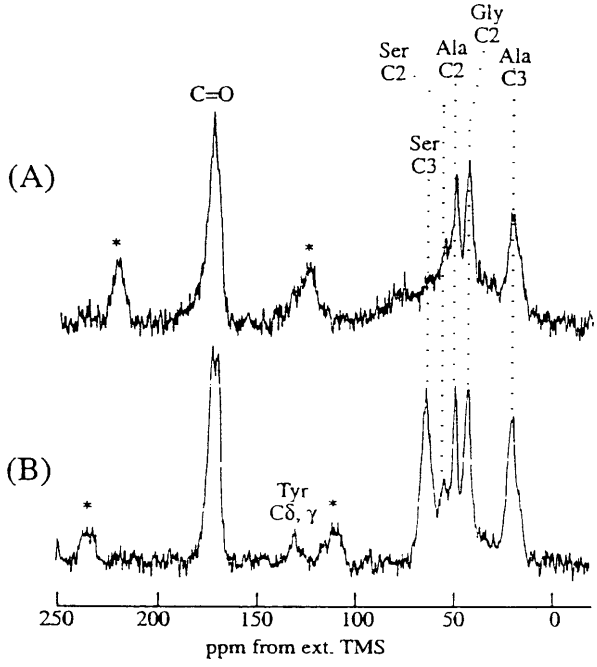

Fig. 3. Solid-state ^{13}C NMR spectra of *B. mori* silk fibroin. **A** Spectrum of natural abundance silk fibroin. **B** Spectrum of silk fibroin labelled by feeding of [^{13}C]formate. The complex peak of C=O consists of C=O peaks of alanine, glycine, serine and tyrosine. *TMS* tetramethylsilane; *spinning site band

fibroin, serine, the third major component in less demand, can serve as a substrate for glycine synthesis (Muramatsu et al., 1961). This interpretation is supported by the fact that ^{13}C of almost all [1-^{13}C]serine given was incorporated into ^{13}C 1 of glycine in fibroin (Fig. 1A). In the posterior silkgland cultured in the folate-deficient medium, a portion of [1-^{13}C]serine administered was converted to glycine (Fig. 1B). It is evident from the results obtained by addition of aminopterin, an inhibitor for folate-related reactions, to the culture medium that H_4F and the derivatives are associated with glycine formation from serine (Fig. 1C). The posterior silkgland contains an amount of H_4F sufficient for this conversion, and this reaction equilibrium may be inclined toward glycine formation.

As shown in animals such as rat and fowl, yeast and higher plants (Ichihara and Greenberg, 1957; Hansford and Davies, 1958; Schramm, 1958; Neuhaus and Byrne, 1959), it was found in the silkworm that serine was synthesized via 3-phosphoglycerate from sugar or glucose contained in diet (Koide and Shimura, 1961; Horie and Shinbo, 1985; Asakura et al., 1988). Serine is also produced by conversion from glycine in this insect (Bricteux-Grégoire et al., 1959b; Fukuda, 1960b). It was reported in rat that formate participates as the C1 donor in conversion of glycine to serine (Sakami, 1949; Siekevitz et al., 1949). Moreover, it was confirmed in yeast and animal tissues (Pasternack et al., 1992; Thureen et al., 1995; Cowin et al., 1996), that H_4F and its derivatives act as necessary cofactors for the conversion (Fig. 2).

In the silkworm also, fibroin obtained by administration of ^{13}C-formate showed specific incorporation to the site C3 of serine (Fig. 3). It was clarified by culture of the posterior silkgland, that, for fibroin synthesis, its cells utilized not only amino acids derived from hemolymph but also self-produced glycine and serine, and performed quantitative regulation between these two amino acids by this interconversion. It was confirmed that this interconversion consisted of metabolic reactions associated with H_4F and its derivatives as co-substrates. There is another major pathway for glycine synthesis via glyoxylate in the silkworm (Koide et al., 1956; Muramatsu and Shimura, 1962), but Muramatsu et al. (1961) assumed by comparison of the incorporation of labelled serine and glyoxylate, that serine seemed to be a more important substrate of glycine synthesis than glyoxylate. Undoubtedly, the glycine-producing system from serine, which is supplied efficiently through glycolysis, must be a potent pathway of serine biosynthesis.

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