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Glutamine Synthetase of Pseudomonads: Some Biochemical and Physicochemical Properties

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The glutamine synthetases from several *Pseudomonas* species were purified to homogeneity, and their properties were compared with those reported for the enzymes from *Escherichia coli* and other gram-negative bacteria. The glutamine synthetase from *Pseudomonas fluorescens* was unique because it was nearly precipitated quantitatively as a homogeneous protein during dialysis of partially purified preparations against buffer containing 10 mM imidazole (pH 7.0) and 10 mM $MnCl_2$. The glutamine synthetases from *Pseudomonas putida* and *Pseudomonas aeruginosa* were purified by affinity chromatography on Affi-blue gel. Dodecamerous forms of the *E. coli* and *P. fluorescens* glutamine synthetases had identical mobilities during polyacrylamide gel electrophoresis. Their dissociated subunits, however, migrated differently and were readily separated by electrophoresis on polyacrylamide gels containing 0.1% sodium dodecyl sulfate. This difference in subunit mobilities is not related to the state of adenylylation. Regulation of the *Pseudomonas* glutamine synthetase activity is mediated by an adenylylation-deadenylylation cyclic cascade system. A sensitive procedure was developed for measuring the average number of adenylylated subunits per enzyme molecule for the glutamine synthetase from *P. fluorescens*. This method takes advantage of the large differences in transferase activity of the adenylylated and unadenylylated subunits at pH 6.0 and of the fact that the activities of both kinds of subunits are the same at pH 8.45.

The glutamine synthetase (GS) activity of *Escherichia coli* is regulated by repression and depression, by feedback inhibition, and by a posttranslational modification which involves an adenylylation of 1 to 12 of the subunits (20). When *E. coli* is grown under conditions of nitrogen excess (high concentrations of NH_4^+), synthesis of GS is repressed and the protein exists in an adenylylated physiologically inactive form (13, 16). Conversely, under NH_4^+-limited growth conditions, biosynthesis of the enzyme is derepressed, and most of the GS subunits are in the unadenylylated (active) state.

This scheme of regulation also exists in several other *Enterobacteriaceae*, including *Salmonella typhimurium* (5), *Klebsiella aerogenes* (1, 5), *Shigella flexneri* (5), and other gram-negative bacteria such as *Azotobacter vinelandii* (10, 18), *Rhizobium* spp. (12), and *Rhodopseudomonas capsulata* (9).

Biochemical and immunological studies (25) suggest that the *Pseudomonas aeruginosa* and *Pseudomonas putida* GSs may also be regulated by similar mechanisms. The two *Pseudomonas* enzymes cross-react with an antiserum prepared against the *E. coli* enzyme, and snake venom phosphodiesterase is able to modify activity of the *Pseudomonas* enzymes (16). However, the existence of adenylylated forms of these enzymes has never been reported.

These considerations and preliminary studies suggesting that the adenylylation of the GS in *P. fluorescens* responds atypically to nitrogen starvation (J. M. Meyer and E. R. Stadtman, unpublished data), prompted a more detailed investigation of the GSs from three major species of psuedomonads: *P. aeruginosa*, *P. putida*, and *P. fluorescens*. This paper reports the isolation of the enzyme from *P. fluorescens* and describes the physicochemical characteristics of the adenylylated and deadenylylated forms of the enzymes from *P. fluorescens*, *P. putida*, and *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. fluorescens* (strain 2798 of the Czechoslovakia Collection of Microorganisms), *P. aeruginosa* (ATCC 15692), and *P. putida* (ATCC 12633) were used throughout the experiments. Growth was at 25°C in 1-liter Erlenmeyer flasks which contained 500 ml of

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medium and which were incubated in a rotary shaker. For nitrogen starvation, the growth medium (pH 7.0) contained 34.4 mM K_2HPO_4, 22 mM KH_2PO_4, 0.81 mM $MgSO_4$, and 33.9 mM succinic acid. Before inoculation with 1% (vol/vol) of a 24-h culture grown under the same conditions, sterile solutions of $FeCl_3$ and $(NH_4)_2SO_4$ were added to final concentrations of 1.6 μM $FeCl_3$ and 2.5 mM NH_4^+, respectively. To obtain large quantities of cells for the enzyme purification, the NH_4^+ concentration of the medium was increased to 10 mM. The cells were usually harvested at the beginning of the stationary phase of growth (40 h).

Buffer mixtures. Buffer A contained 40 mM imidazole hydrochloride (pH 7.0), 10 mM $MgCl_2$, and 0.1 M KCl. Buffer B was the same as buffer A but with 0.3 M KCl. Buffer C was the same as buffer A but with 1.5 M KCl. Buffer D contained 30 mM Tris-hydrochloride (pH 8.0), 10 mM $MgCl_2$, and 1 mM $MnCl_2$. Buffer E contained 10 mM imidazole hydrochloride (pH 7.0) and 1 mM EDTA-Mg. Mixed imidazole buffer was prepared as previously described (22). The TEA-DMG buffers were prepared by titrating dimethylglutaric acid (DMG) with triethanolamine (TEA) to the desired pH.

Assay of GS. Enzyme activity was measured by the γ-glutamyltransferase method described previously for assay of the *E. coli* enzyme (17), except that the pH of the assay mixture was 8.45 instead of 7.57. This change in pH was necessary because adenylylated and unadenylylated forms of the *P. fluorescens* enzyme have identical activities at pH 8.45 rather than at 7.57, as occurs with the *E. coli* enzyme. One unit of enzyme activity is defined as 1.0 μmol of glutamate hydroxamate formed (absorbance at 540 nm, 0.360) per min at 37°C. Protein concentration was determined by the method of Bradford (3), with bovine serum albumin (BSA) as a standard. The average number, $\bar{n}$, of adenylylated subunits per enzyme molecule (e.g., $GS_{\overline{12}}$), referred to as the state of adenylylation, was calculated from measurements of the γ-glutamyltransferase activity at pH 8.45 (as described above) and at pH 7.15 (see below).

Anti AMP-BSA antiserum precipitation. As described previously (7), increasing amounts (0 to 200 μl) of anti-AMP-BSA antiserum, kindly provided by R. J. Hohman, were added to samples containing 36 μg of *P. fluorescens* GS in 0.5 ml of buffer A. After 30 min at 37°C and 18 h at 4°C, the samples were centrifuged (10 min, 5,000 × *g*), and the enzyme left in the supernatants was measured by the transferase assay at pH 8.45.

Snake venom phosphodiesterase treatment. Snake venom phosphodiesterase (39 U/mg) was obtained from Worthington Diagnostics. Before use, it was further purified by being passed through a concanavalin A-Sepharose column (24). To prepare unadenylylated enzyme, 16 mg of *P. fluorescens* GS in 2.0 ml of buffer B was diluted 10 times with buffer D, mixed with 15 U of snake venom phosphodiesterase, and incubated at 37°C. The reaction was stopped when the transferase activity, as measured at pH 7.15 in the TEA-DMG-buffered system, reached a constant minimal value, which was unchanged even after more fresh snake venom phosphodiesterase was added. To separate GS and snake venom phosphodiesterase, the protein solution was concentrated to 6 ml by ultrafiltration (Amicon filter XM50), loaded onto a concanavalin A-Sepharose column (5 by 1 cm), and eluted with 0.2 M sodium acetate (pH 6). Fractions containing GS activity were pooled, dialyzed against buffer B, and concentrated by ultrafiltration. Recovery of the enzyme was about 70%. To demonstrate that AMP was released from GS by treatment with snake venom phosphodiesterase, the filtrate obtained by ultrafiltration was concentrated by evaporation under reduced pressure and passed through a Norite column (1 by 1 cm). The column was washed with distilled water and then eluted with 10 ml of solution containing 50% ethanol, 50% water, and NH_4OH to pH 10. The eluate was lyophilized, and the dry residue was redissolved in 2 ml of distilled water and then analyzed by thin-layer chromatography on polyethyleneimine-cellulose, using 1 M LiCl as the developing solvent. The putative AMP sample was compared with standards containing 3 μg each of AMP, CMP, GMP, and UMP. The location of the nucleotides was visualized by UV light.

Adenylyltransferase reaction. To prepare fully adenylylated GS, the unadenylylated enzyme, obtained by snake venom phosphodiesterase treatment (see above), was dialyzed overnight at 4°C against buffer E. Adenylylation of the dialyzed enzyme was obtained by incubating it with a partially purified adenylyltransferase preparation from *E. coli* (kindly provided by S. G. Rhee) under the previously described adenylylation conditions (15).

Gel electrophoresis. Tube gel electrophoresis (8% polyacrylamide) of native proteins was performed by the method of Davis (4). The protein bands were visualized with 0.04% Coomassie blue G250 in 3.5% perchloric acid. On duplicate gels, GS was located by its glutamyltransferase activity, as previously described (14). Slab gel electrophoresis on sodium dodecyl sulfate-polyacrylamide (10%) gels was as described by Laemmli (11). The proteins used for molecular weight standardization were phosphorylase b, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

RESULTS

Purification of GS of *P. fluorescens*. Attempts to purify GS from *P. fluorescens* by the method of Woolfolk et al. (27) or by the zinc precipitation method of Miller et al. (14), originally used in the purification of *E. coli* enzyme, were unsuccessful. The yield in both methods was less than 10%; a 30% loss occurred during the first streptomycin step. A more efficient method for the purification of the *P. fluorescens* enzyme was developed by taking advantage of the heat resistance of this enzyme and its insolubility in a low-ionic-strength environment in the presence of manganese.

Step 1: crude extract preparation. Twenty grams of cells (wet weight), grown as described above, were suspended in 50 ml of buffer A, adjusted to pH 7.2, and passed twice through a French press. Before centrifugation (10 min at

30,000 × *g*, 4°C), the extract was sonicated for three 30-s pulses to decrease the viscosity of the preparation.

Step 2: heat treatment. The supernatant of step 1 was incubated for 30 min in a water bath at 63°C, with intermittent shaking. The material was then centrifuged for 10 min at 30,000 × *g*, and the precipitate washed once with the extraction buffer. The two supernatant fractions were pooled for the next step.

Step 3: ammonium sulfate precipitation. Solid ammonium sulfate was added to the enzyme solution to 60% saturation, and after 30 min at room temperature, the mixture was centrifuged at 30,000 × *g* for 10 min. The supernatant was discarded, and the precipitate was dissolved in 20 ml of the extraction buffer.

Step 4: acetone precipitation. The dissolved precipitate from step 3 was brought to room temperature, and acetone was slowly added to a final concentration of 45% by volume. After 5 min, the precipitate was recovered by centrifugation and suspended in 10 ml of the extraction buffer. After 2 h of being gently stirred at 4°C, the suspension was centrifuged and the insoluble material was discarded.

Step 5: dialysis. The enzyme solution from step 4 was dialyzed overnight at 4°C against 2 liters of pH 7.0 buffer containing 10 mM imidazole and 10 mM $MnCl_2$ (no KCl). Under these conditions, the GS precipitated in the dialysis sac and was recovered by centrifugation after one washing with the imidazole-$MnCl_2$ buffer. The precipitated enzyme was redissolved in buffer B. After step 5, the enzyme appeared to be homogeneous; only one band was obtained on polyacrylamide electrophoresis in the presence or absence of sodium dodecyl sulfate. The purified enzyme could be stored at 4°C for several months without losing activity.

The yield and specific activity of the enzyme after the various purification steps are summarized in Table 1. Routinely, between 13 and 15 mg of pure enzyme was recovered from 20 g of starting material.

GS of *P. aeruginosa* and *P. putida* failed to precipitate by dialysis as in step 5. For these two enzymes, the extracts obtained after step 4 were purified by chromatography on Affi-blue gel (column [4 by 0.6 cm] equilibrated with buffer C; the enzyme eluted with 10 mM ADP in the same buffer). This last procedure was not used for the *P. fluorescens* enzyme because the recovery was only 20 to 30%. Chromatography on Affi-blue gel has been used successfully in the purification of GS from *Azotobacter vinelandii* (18), cyanobacteria (19), and *E. coli* (21).

TABLE 1. *Purification of P. fluorescens GS*

Protein fraction	Sp act[a]	Yield (%)
Crude extract	0.9	
Heat treatment	4.0	82.5
$(NH_4)_2SO_4$ precipitation	6.0	68.7
Acetone precipitation	14.7	67.8
Dialysis precipitation	47.3	52

[a] The specific activity, expressed in units of GS per milligram of protein, refers to the transferase activity at pH 8.45 in a TEA-DMG-buffered system of measurement (see text).

Immunoprecipitation with anti-AMP-specific antibodies. Previous studies (7) showed that the fraction of *E. coli* GS that can be precipitated by anti-AMP-specific antibodies is proportional to the average number of adenylylated subunits per dodecamer; no precipitate occurs with unadenylylated enzyme, whereas fully adenylylated enzyme is quantitatively precipitated. The *P. fluorescens* enzyme isolated from ammonia-rich medium (10 mM NH_4^+) was almost completely precipitated by the anti-AMP antibodies (Fig. 1, lower curve), whereas no precipitation occurred when the enzyme was first treated with snake venom phosphodiesterase to remove covalently bound adenylyl groups (Fig. 1, upper curve). The antibodies precipitated only 50% of another enzyme preparation which was isolated from cells also grown in the 10 mM NH_4^+ medium but harvested earlier, during the exponential phase of growth (Fig. 1, middle curve). By analogy to the immunoprecipitation patterns of *E. coli* GS preparations, it may be concluded that the upper, middle, and lower curves in Fig. 1 correspond to GS preparations containing 12, 6, and 0 covalently bound adenylyl groups, respectively.

pH profiles of *Pseudomonas* GS. As was demonstrated for the *E. coli* enzyme (20, 22), the pH profile of the *Pseudomonas* GS varied with the average state of adenylylation. The fully adenylylated enzyme exhibited maximum γ-glutamyltransferase activity at pH 7.15 in the TEA-DMG-buffered system, whereas unadenylylated enzyme exhibited only about 10% as much activity at this pH (Fig. 2). All forms of the enzyme had the same activity at pH 8.45 (the isoactivity pH). The γ-glutamyltransferase activity observed at pH 8.45 was, therefore, a measure of the total amount of enzyme present, regardless of the state of adenylylation. Other studies showed that pH 8.45 was also the isoactivity pH of the γ-glutamyltransferase in crude extracts of *P. fluorescens*.

For these studies, extracts containing GS with both low and high states of adenylylation were obtained by the NH_4^+ shock procedure of Jans-

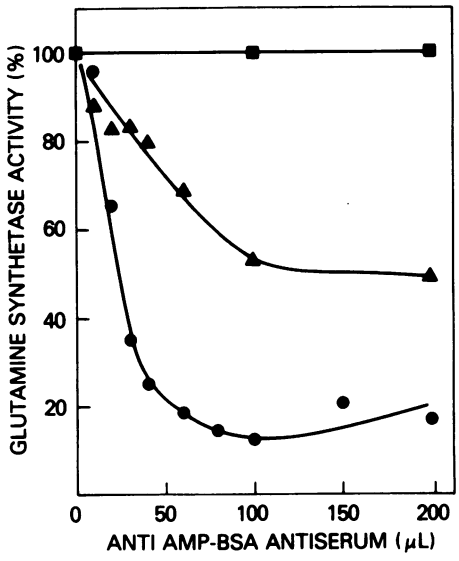

FIG. 1. *Precipitation of P. fluorescens GS preparations by anti-AMP antiserum. The concentration of antiserum formed against adenylylated BSA was varied from 0 to 200 μl as indicated. Symbols: ●, GS purified from stationary-phase cells grown on 10 mM NH_4^+; ■, GS from stationary-phase cells grown on 10 mM NH_4^+ after treatment with snake venom phosphodiesterase and repurification; ▲, GS purified from exponential-phase cells grown on 10 mM NH_4^+. The activity was measured by the transferase assay at pH 8.45 in TEA-DMG buffer.*

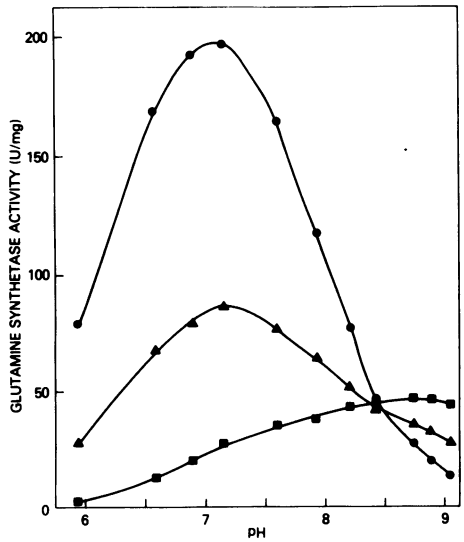

FIG. 2. *The pH profiles of the transferase activity (TEA-DMG buffer) of purified P. fluorescens GS ($GS_{\bar{n}}$) preparations with different states of adenylylation ($\bar{n}$). Symbols: ●, $GS_{\overline{12}}$; ▲, $GS_{\overline{5.3}}$; ■, $GS_{\overline{0.8}}$.*

sen and Magasanik (8). Stationary-phase cultures (in NH_4^+-deficient medium, 2.5 mM NH_4^+) were divided into two equal parts, one of which was supplemented with 20 mM $(NH_4^+)_2SO_4$ 5 min before harvesting. The cells were harvested by centrifugation, and the cell-free extracts were prepared by procedures (to be described elsewhere) that prevent further changes in the state of adenylylation. As expected from the results of Janssen and Magasanik (8), the NH_4^+-treated and nitrogen-starved cells exhibited pH profiles characteristic of the adenylylated and unadenylylated enzymes, respectively (isoactivity pH 8.45).

The NH_4^+ shock procedure was also used to determine the isoactivity pH (transferase assay) for the *P. aeruginosa* and *P. putida* enzymes. The pH activity profiles of GS from both organisms were similar to that observed for *P. fluorescens*; however, the pH optima as well as the isoactivity pH of all three organisms were slightly different. Thus, with the TEA-DMG buffer system, the isoactivity pH values of *P. fluorescens*, *P. aeruginosa*, and *P. putida* were 8.45, 8.75, and 8.85, respectively, and the pH optima were 7.15, 7.37, and 7.14, respectively. In a mixed imidazole buffer system (22), the corresponding isoactivity pH values were 8.10, 8.35, and 8.30, respectively, and the pH optima were 6.85, 7.35, and 7.15, respectively. The pH activity profiles with the latter buffer were shifted to lower pH values but were otherwise qualitatively similar to those observed with the TEA-DMG buffer.

Determination of the state of adenylylation of GS of *P. fluorescens*. (i) Measurement of transferase activity at pH 7.15 and 8.45 in TEA-DMG buffer. It is evident that at pH 8.45, the transferase activity of GS was independent of the state of adenylylation, whereas at pH 7.15, the activity depended on the amount of enzyme present and the state of adenylylation (Fig. 2). The ratio of activity at pH 7.15 (where the adenylylated subunits are the more active) to the activity at pH 8.45 is therefore proportional to the state of adenylylation. For fully unadenylylated enzyme obtained by treatment with snake venom phosphodiesterase, this ratio is 0.36, whereas for the fully adenylylated GS, the ratio is 4.12.

From these values, it can be shown that the average state of adenylylation ($\bar{n}$) of the enzyme is given by the formula $\bar{n} = [3.2(x/y)] - 1.2$, where x and y refer to the transferase activities measured at pH 7.15 and 8.45, respectively.

Validity of this calculation was indicated by the fact that a preparation only half of which was precipitated by the anti-AMP antibodies

(middle curve, Fig. 1) yielded an $\bar{n}$ value of 6.0. Ten determinations gave values from 5.3 to 6.5 by this formula.

(ii) ADP ratio method. From double-reciprocal plots of transferase activity versus ADP concentration (pH 8.45), it was demonstrated that the apparent K_m values of unadenylylated and adenylylated subunits for ADP were 0.04 and 4.0 μM, respectively. From the measured K_m values, it can be calculated that at a concentration of 1 μM ADP in the assay mixture (TEA-DMG buffer), the adenylylated enzyme subunits will be 20% saturated with ADP, whereas the unadenylylated subunits will be 96% saturated. Therefore, the state of adenylylation can be calculated by the expression $\bar{n} = 15.15 - 15.80\ y$, where y is the ratio of the transferase activity at pH 8.45 in the TEA-DMG buffer system with 1 μM ADP to the activity under the same conditions but with 400 μM ADP (saturating condition).

In vitro adenylylation and deadenylylation of the *P. fluorescens* GS. When the GS isolated from cells grown in nitrogen-rich medium (10 mM NH_4^+) was incubated with snake venom phosphodiesterase, the γ-glutamyltransferase activity as measured at pH 7.15 declined to a low constant value, whereas the activity at pH 8.45 (isoactivity point) remained constant. Variations in the ratio of these activities correspond to a change in the adenylylation state from 11 to 1.0 in a 2-h experiment (Fig. 3). At the end of this experiment, AMP was isolated from the incubation mixtures, as described above. It is therefore evident that, as was demonstrated for the *E. coli* enzyme (16), the GS from cells grown in a nitrogen-rich medium exists in a highly adenylylated state and that the adenylyl groups can be removed by snake venom phosphodiesterase.

Other data in Fig. 3 (closed circles) show that when unadenylylated *P. fluorescens* GS isolated from nitrogen-starved cells was incubated with a partially purified preparation of adenylyltransferase from *E. coli* (15) and an ATP-generating system, the state of adenylylation increased from about 0.5 to 11.0.

Mobility of GS of *Pseudomonas* spp. on gel electrophoresis. The purified enzyme preparations obtained from *P. fluorescens*, *P. aeruginosa*, and *P. putida* were analyzed on native polyacrylamide gels by using the Davis system (4), with *E. coli* enzyme as a standard. Visualization of the native protein on the gels with Coomassie blue by the activity staining technique (see above) revealed only one protein band in each case, which migrated exactly at the same place that the *E. coli* GS migrated (data not shown).

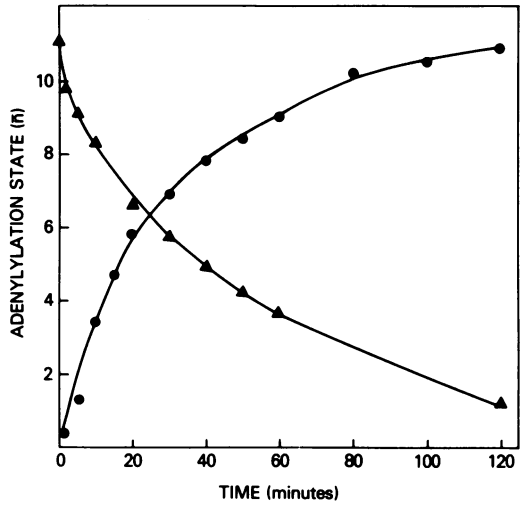

FIG. 3. *Variations of the adenylylation state ($\bar{n}$) of P. fluorescens GS by treatment of the fully adenylylated enzyme with snake venom phosphodiesterase or by treatment of unadenylylated enzyme with adenylyltransferase from E. coli. The assay for snake venom phosphodiesterase treatment contained (in a final volume of 200 μl of buffer D) 75 μg of adenylylated GS ($GS_{\overline{11}}$) and 0.2 U of snake venom phosphodiesterase. Temperature of incubation was 37°C. The 1-ml assay mixture for adenylyltransferase reaction contained 20 mM imidazole (pH 7.5), 0.3 M KCl, 210 μg of unadenylylated GS, 20 mM $MgCl_2$, 1 mM ATP, 2 mM phosphoenolpyruvate, 10 μl of pyruvate kinase (38 U), and 20 mM glutamine. A crude preparation (10 μl) of adenylyltransferase from E. coli (15) was added before glutamine and the reaction mixture was incubated at 37°C. At the times indicated, 10-μl portions of the incubation mixtures were diluted 10-fold with buffer A and kept on ice until the transferase activities at pH 7.15 and 8.45 were measured. Symbols: ▲, snake venom phosphodiesterase reaction; ●, adenylyltransferase reaction.*

However, with slab gel electrophoresis in 10% polyacrylamide, containing 0.1% sodium dodecyl sulfate, the subunits of the three *Pseudomonas* enzymes migrated in identical manners that were significantly different from that obtained with the *E. coli* enzyme. As can be seen in Fig. 4, subunits of *Pseudomonas* and *E. coli* GS are very well separated. By comparing their mobilities with standard proteins, an apparent molecular weight of 62,000 was calculated for *P. fluorescens* and *P. aeruginosa* GS subunits, and 61,000 was calculated for the *P. putida* subunit. In this system, the *E. coli* GS subunit, which was reported to have a molecular weight of 50,000 (20), has an apparent molecular weight of 56,500. This last result is in agreement with the value previously reported by Bender and Streicher (2), who calculated an apparent molecular weight of 57,000 for the *E. coli* subunit

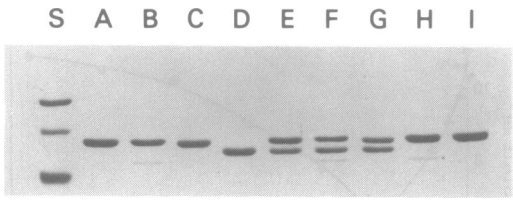

FIG. 4. *Comparative mobilities during sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis of various GS preparations. Preparations as follows: (A) P. fluorescens, (B) P. aeruginosa, (C) P. putida, (D) E. coli, (E, F, G, H, and I) equal mixtures of A + D, B + D, C + D, A + B, and A + C, respectively. Each slot was loaded with 3 μg of protein. (S) Molecular weight standard proteins, top to bottom: phosphorylase b (94,000), BSA (68,000), and ovalbumin (48,000).*

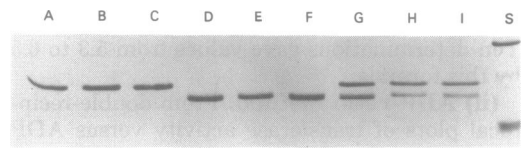

FIG. 5. *Comparative mobility of adenylylated and unadenylylated subunits from GS of P. fluorescens and E. coli. Each slot was loaded with a total of 3 μg of protein. (A) Unadenylylated GS of P. fluorescens ($GS_{\overline{1}}$); (B) adenylylated GS of P. fluorescens ($GS_{\overline{12}}$); (C) equal amounts of A + B; (D) unadenylylated GS of E. coli ($GS_{\overline{2}}$); (E) adenylylated GS of E. coli ($GS_{\overline{12}}$); (F) equal amounts of D + E; (G) equal amounts of A + D; (H) equal amounts of B + E; (I) equal amounts of A + B + D + E; (S) molecular weight standard proteins, top to bottom: phosphorylase b (94,000), BSA (68,000), ovalbumin (48,000).*

in the same Tris-buffered system of gel electrophoresis. Figure 5 shows the electrophoretic pattern of both adenylylated ($GS_{\overline{12}}$) and unadenylylated ($GS_{\overline{1\text{-}2}}$) forms of *P. fluorescens* and *E. coli* GS. The mobilities of adenylylated and unadenylylated subunits were identical for both enzymes. Therefore, the slower migrations of the *Pseudomonas* enzymes, compared with the *E. coli* GS, are due to a higher molecular weight and not to a difference in the state of adenylylation.

DISCUSSION

From the results of immunodiffusion studies with antibodies prepared against *E. coli* GS, Tronick et al. (25) ordered the GS from different microorganisms according to antigenic similarity, as follows: *E. coli* = *S. typhimurium* = *K. pneumoniae* > *Serratia marcescens* > *Proteus mirabilis* > *P. putida* = *P. aeruginosa* = *A. vinelandii* > *Bacillus polymyxa* > *Rhizobium japonicum*. According to this order, the GS from *Pseudomonas* spp. is much more closely related to the GS from *A. vinelandii* than to the GS from *E. coli* or *S. typhimurium*. This order of relatedness is also suggested by comparisons of the pH activity profiles, molecular weights, and kinetic parameters.

The pH activity profiles of the adenylylated forms of GS from the three pseudomonads examined here are similar to those observed with *E. coli* (22) and *A. vinelandii* (18). The profiles of the unadenylylated *Pseudomonas* enzymes are significantly different from that of the unadenylylated *E. coli* enzyme, but are significantly similar to the profile of the unadenylylated *A. vinelandii* enzyme. Furthermore, the isoactivity pH values of the *P. fluorescens* and *A. vinelandii* enzymes are similar (8.45 and 8.60, respectively), but are much higher than the isoactivity pH of 7.57 for the *E. coli* enzyme (22).

Owing to the marked difference in these isoactivity points, the methods developed for determination of the average state of adenylylation of the *E. coli* enzyme (22) cannot be used to determine the state of adenylylation of the *Pseudomonas* enzyme. However, by taking advantage of the facts that at pH 7.15 the adenylylated enzyme is about eight times more active than the unadenylylated enzyme and that at pH 8.45 the activity is independent of the state of adenylylation, a method was developed (see above) for determining the average state of adenylylation of the enzyme from *P. fluorescens*. With only slight modifications to correct for slight shifts in the isoactivity pH, the method is applicable also to the enzymes from *P. putida*, *P. aeruginosa*, and *A. vinelandii*.

By electron microscopy, it has been established that the GS from *E. coli* (26), *A. vinelandii* (18), and *P. fluorescens* (A. Segal, E. Shelton, and E. R. Stadtman, unpublished data) are all composed of 12 apparently identical subunits arranged in two hexagonal arrays. The native enzyme from all species migrates with similar mobilities during the disc gel electrophoresis; however, in the presence of sodium dodecyl sulfate, subunits of the *E. coli* GS migrate faster and are readily separated from subunits of the GS from either *P. fluorescens* (this report) or *A. vinelandii* (18). These results agree with those of Streicher and Tyler (23), showing that pure preparations of GS from *A. vinelandii* (18) and *Klebsiella* have identical mobilities which are slower than that of the *E. coli* enzyme and which are identical to one of two major protein bands in a partially purified preparation of the enzyme from *P. putida*. In light of the present results, it

is likely that the slower of the two protein bands in the Streicher and Tyler preparation of *P. putida* corresponds to the GS. The apparently higher molecular weights of the *P. fluorescens* and *A. vinelandii* GS subunits compared with that of the *E. coli* enzyme are probably not attributable to the presence of glycosyl groups, since neither enzyme is retarded on concanavalin A-Sepharose columns. Moreover, the differences in mobilities are not due to differences in the states of adenylylation, since under the condition of electrophoresis used here, the adenylylated and unadenylylated subunits of GS from either *E. coli* or *P. fluorescens* could not be separated from one another, as they could be according to Bender and Streicher (2) and Goldberg and Hanau (6). We have repeatedly tried and failed to detect separation of adenylylated and unadenylylated subunits of the *E. coli* GS, following as nearly as possible the procedure described by Bender and Streicher (2). We have no explanation for the discrepancy between their results and ours.

Finally, we note that the apparent K_m of the adenylylated form of the *P. fluorescens* enzyme for ADP (4 μM at pH 8.45) is about an order of magnitude lower than that for the *E. coli* enzyme (30 μM at pH 7.57). Whether this difference reflects intrinsic characteristics of the two enzymes or is a manifestation of the measurements being made at different pH levels (the respective isoactivity pH values) has not been determined.

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