Regulatory Properties of Glutamine Synthetase from the Nitrogen-Fixing Phototrophic Bacterium *Rhodopseudomonas palustris*

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Glutamine synthetase from *Rhodopseudomonas palustris* is regulated *via* an adenylylation/deadenylylation mechanism. The enzyme purified from ammonia-grown cells, released AMP upon treatment with phosphodiesterase, along with drastic changes in its pH and metal dependency. Kinetic parameters for enzyme-substrate interaction were also dependent on the adenylylation state of the enzyme, as was the influence of several nitrogenous feedback inhibitors on the catalytic activity. The adenylylation state of the enzyme was modified *in vivo* by the availability of ammonia.

Introduction

Nitrogen-fixing or ammonia-utilizing cells of the photosynthetic purple bacterium *Rhodopseudomonas palustris* assimilate ammonium via the enzyme glutamine synthetase (EC 6.3.1.2, L-glutamate: ammonia ligase [ADP forming]) [1]. In a previous article we have described purification of this enzyme to apparent homogeneity and have reported on its basic molecular properties [1]. Glutamine synthetase has been studied from several microorganisms and has been shown to be subject to intricate regulation, involving repression and derepression, metabolic interconversion between two covalently modified forms, cumulative feedback inhibition, and response to divalent cations [2]. In this communication we describe evidence for part of those regulatory principles for the glutamine synthetase from *R. palustris*. A covalent control mechanism has been inferred previously by the action of phosphodiesterase on a partially purified preparation of glutamine synthetase from *Rhodopseudomonas capsulata* [3]. We have studied the regulatory properties of glutamine synthetase from *R. palustris* in more detail, because of its possible involvement in the regulation of nitrogenase at the level of enzyme synthesis and activity. A striking observation within the latter aspect is the absence of inhibition of nitrogenase by ammonia when glutamine synthetase is blocked by the transition-state analog methionine sulfoximine. This effect has been demonstrated for *R. palustris* [4] as well as for several other phototrophic bacteria [5–7].

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Materials and Methods

Organism and growth conditions

Rhodopseudomonas palustris (ATCC 17001) was grown on 15 mM NH_4Cl as N-source as previously described [1] in completely filled 1 l screw-cap bottles (Roux type). The growth medium contained per liter 4 g malate, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot 5H_2O$, 1.02 g KH_2PO_4, 5 mg Fe-citrate, 10 ml of trace element solution SL_6 [8], and 0.5 g yeast extract (Difco). The medium was adjusted to pH 6.9 with NaOH prior to autoclaving. Before harvest, the cells were treated with cetrimide to stabilize the state of adenylylation [1], since otherwise only intermediate degrees were obtained [15].

Enzyme source

Glutamine synthetase was highly purified by dye-ligand and ion exchange chromatography as previously detailed [1]. A predominantly adenylylated enzyme was obtained from ammonia-grown cells. Where a deadenylylated enzyme was required, a dialyzed enzyme sample was treated for 60 min with phosphodiesterase similar to the procedure described under Analytical Methods.

Determination of the relative degree of adenylylation in whole cells

A cell suspension in 45 mM phosphate buffer, pH 6.9, was incubated with dinitrogen in the light (illuminance 10000 lux) under the desired experimental conditions. At time intervals 0.3 ml of the cell suspension was removed by gas-tight syringe and injected into a nitrogen-filled 13 ml vial containing 50 µl of aqueous solution of cetrimide (N-cetyl-N,N,N-trimethylammonium bromide, 15 mg/ml) [15]. The cells were permeabilized by the detergent during an incubation period of 5 min in the light. Afterwards the transferase activity was measured in the presence and absence of 50 mM Mg^{2+} at pH 6.8 by the standard assay [1]. The relative degree of adenylylation is given by the ratio of these activity measurements.

Enzyme assays

The biosynthetic and the transferase activities of gutamine synthetase were measured as specified previously [1].

The synthetic activity was assayed by a minor modification of a published procedure [9]. The reaction mixture contained in a total volume of 1 ml, an enzyme aliquot, 100 µmol 3,3-dimethylglutaric acid, 8 µmol sodium ATP, 100 µmol L-glutamate, 20 µmol hydroxylamine, and 8 µmol $MnCl_2$ or 50 µmol $MgCl_2$. The pH was adjusted to 6.8 for the standard assay with 2-fold diluted triethanolamine or with the same base to other desired pH values for measuring the pH profile. After incubating the reaction mixture at 35 °C for 10–30 min, the reaction was stopped by the addition of 2 ml acidic $FeCl_3$ and the resulting γ-glutamylhydroxamate was measured spectrophotometrically at 500 nm [10].

Analytical methods

AMP liberated from the enzyme by treatment with phosphodiesterase was quantitated by bioluminescence [11, 16]. A sample of purified glutamine synthetase was dialyzed against 3 l of 10 mM Tris-HCl, pH 7.1, containing 1 mM $MnCl_2$. An enzyme aliquot of 0.8 ml with 147 µg protein was incubated with 40 µg phosphodiesterase from snake venom (Sigma) at 35 °C. Samples were removed at regular time intervals and analyzed for transferase activity in the presence of 50 mM $MgCl_2$ and for the presence of free AMP. For the latter purpose a sample of 0.1 ml was deproteinized with 0.1 ml of 35% (w/v) $HClO_4$ and then neutralized by adding 310 µl 2 M $KHCO_3$. The mixture was incubated at 0 °C for 15 min, and then centrifuged in the cold at 23000×g. From the supernatant 0.2 ml was placed in a reaction vial together with 0.1 ml of 2 µM ATP and 5 µl of myokinase, which corresponded to 33 µg. After incubation at room temperature for 20 min, 0.2 ml of a mixture of crude firefly lantern extract (Sigma, FLE-250) was added to 50 µl of the incubation mixture simultaneously with 1.9 ml reaction buffer (10 mM $MgSO_4$ in 0.1 M Tris-HCl, pH 7.4). Maximal light emission was measured with a bioluminescence detector XP 2000 (Skan, Basel, Switzerland) and quantitated with ATP standards.

Ammonia was determined by the Berthelot reaction to indophenol [12]. Protein concentrations of cell-free extracts were measured by the method of Lowry; for whole cells, alkaline digestion and reaction with $CuSO_4$ according to Stickland was used [13]. Bovine serum albumin served as standard for both cases.

Results and Discussion

In vitro interconversion of glutamine synthetase

The glutamine synthetase from *R. palustris* can be expected to have properties similar to that of other Gram-negative bacteria. A presumptively adenylylated enzyme thus would be obtained from ammonia-grown cells. This should become deadenylylated by treatment with phosphodiesterase, as was proven in other bacteria [3, 14–16]. Fig. 1 shows the effect of phosphodiesterase on the transferase activity of and AMP liberation from a purified sample of glutamine synthetase. The transferase activity was strongly inhibited in the presence of 50 mM $MgCl_2$. This inhibition was successively abolished by incubation with phosphodiesterase, which caused concomitantly the liberation of covalently bound AMP moieties. Per mol protein 11.7 mol of AMP were released (Fig. 1), corresponding to 12 subunits of the enzyme [1]. These data demonstrate that the glutamine synthetase from *R. palustris* is covalently modified by adenylyl groups under certain metabolic conditions (here excess of ammonia) and that these groups can be removed enzymatically *in vitro* by splitting a phosphodiester linkage.

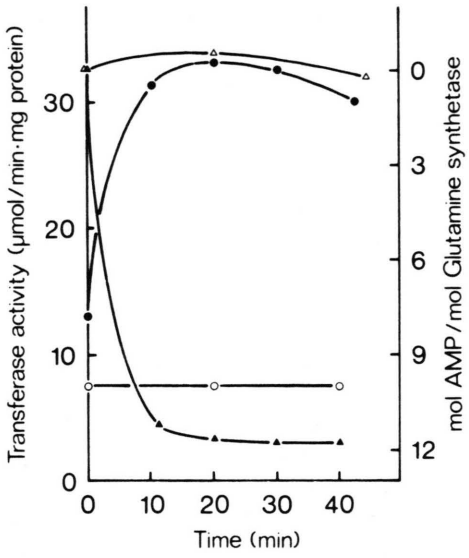

Fig. 1. Effect of phosphodiesterase treatment on a sample of purified glutamine synthetase isolated from an ammonia-grown culture. The transferase activity was measured in the presence of 50 mM Mg^{2+} of treated (●) and untreated (○) enzyme. AMP release from the enzyme (▲) was determined as described under Materials and Methods. A control sample of glutamine synthetase was not treated with phosphodiesterase, but was otherwise carried alike through the entire assay procedure. In this case no AMP was liberated from the enzyme (△).

pH-Profiles

The two forms of glutamine synthetase should show different pH dependencies in their enzymatic activities [17]. We therefore measured the pH profiles of deadenylylated (*i.e.* enzyme treated with phosphodiesterase) and predominantly adenylylated enzyme (*i.e.* enzyme from ammonia-grown cells) with respect to the following catalytic activities: transferase activity, Mn^{2+}-dependent biosynthetic activity, and Mg^{2+}-dependent synthetic activity. Under our experimental conditions we found different responses towards pH and a broad range of nearly equal activity from pH 6.0 to 7.1 when following transferase activity of the adenylylated and deadenylylated forms of glutamine synthetase. Biosynthetic and synthetic activities of the two enzyme forms showed even more divergent responses towards pH, with an isoactivity point at pH 6.45 in the biosynthetic assay only. The findings of these experiments are summarized in Fig. 2.

Specificity for divalent cations

Glutamine synthetase from *Escherichia coli* has an absolute requirement for divalent cations which can be satisfied either by Mg^{2+} or Mn^{2+} [18, 19]. The relationship between Mn^{2+} and ATP for the biosynthetic activity of predominantly adenylylated enzyme from *R. palustris* is shown in Fig. 3A. An increase of the Mn^{2+}-concentration in the assay at each of three ATP levels studied (4, 8, and 15 mM) resulted in maximal catalytic activity at a ratio of cation to nucleotide of one. Marked inhibition was seen when either Mn^{2+} or ATP were in excess. The $MnATP^{2-}$ complex thus appears to be the active substrate for glutamine synthetase. In contrast to the Mn^{2+}-dependent reaction, maximal catalytic activity in the presence of Mg^{2+} was found only when this cation was in large excess over ATP (Fig. 3B). The activity saturated around a six-fold excess of Mg^{2+} and higher ratios did not affect the enzyme.

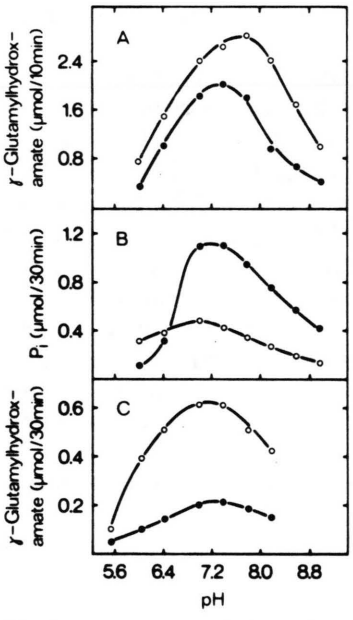

Fig. 2. pH-Activity profiles of partially adenylylated (●) and deadenylylated (○) glutamine synthetase purified from *R. palustris*. A) Profile for the transferase reaction. The buffer was 62.5 mM 3,3-dimethylglutaric acid/triethanolamine. The reaction mixture contained 3.2 µg of enzyme. B) Profile for the Mn^{2+}-dependent biosynthetic activity followed as release of inorganic phosphate (P_i). The reaction mixture contained 16 µg protein and a mixed buffer of 0.25 M maleic acid, 0.25 M imidazole, and 0.25 M Tris, adjusted to the different pH values. C) Profile for the Mg^{2+}-dependent synthetic activity. Buffer same as under (A), but 100 mM; glutamine synthetase 11 µg.

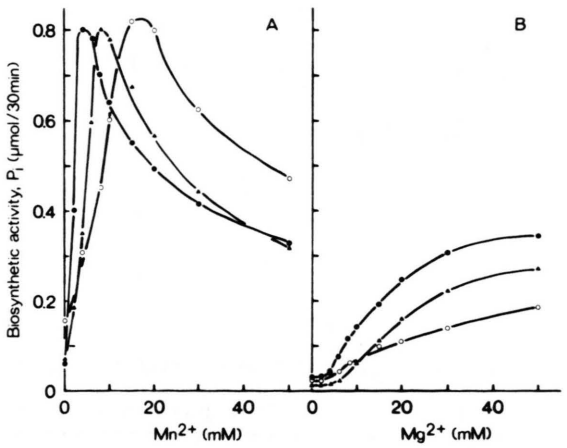

Fig. 3. Dependency of the biosynthetic activity of adenylylated glutamine synthetase on manganese and magnesium. Three levels of ATP were used: (●) 4 mM, (▲) 8 mM, and (○) 15 mM. Glutamate was at 100 mM, and ammonia at 50 mM. The reaction mixture contained 14.5 µg of protein, and the reaction was followed at pH 7.0. A) Dependency on Mn^{2+}; maximal activities were at 4.5 mM, 8 mM and 16.5 mM Mn^{2+}; B) dependency on Mg^{2+}.

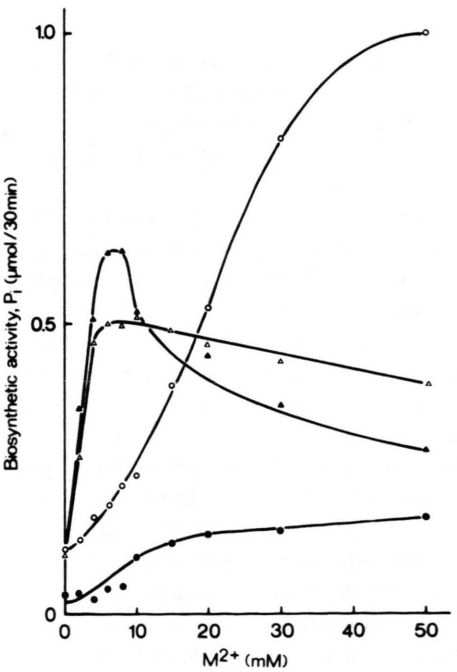

Fig. 4. Relationship between cation concentration and the adenylylation state of *R. palustris* glutamine synthetase followed as biosynthetic activity. Triangles represent Mg^{2+}-dependent phosphate release (P_i) from ATP by adenylylated (▲) and deadenylylated (△) enzyme. Circles represent the same Mg^{2+}-dependent activity by adenylylated (●) and deadenylylated (○) enzyme. The assays contained 7.4 µg of glutamine synthetase, 100 mM glutamate, 50 mM ammonia, and 8 mM ATP.

The role of divalent cations in the biosynthetic reaction of glutamine synthetase from *R. palustris* was further studied in response to the adenylylation state of the enzyme. As shown in Fig. 4, the adenylylated form of glutamine synthetase catalyzed the biosynthetic reaction more rapidly in the presence of Mn^{2+}, while the deadenylylated enzyme was more active in the presence of Mg^{2+}. Maximal activity of the deadenylylated form required again about a six-fold excess of Mg^{2+} over the nucleotide as was shown above for the adenylylated form. In this experiment an ATP concentration of 8 mM was used and both forms of glutamine synthetase showed maximal activity at this concentration of Mn^{2+}; the deadenylylated form, however, had a slightly lower maximal activity and did not show the pronounced inhibition when the cation was in excess of the nucleotide (Fig. 4). These data manifest the interaction of the adenylylation state of the enzyme with available cations for a fine tuning of the enzymatic activity.

Apparent K_m values

Substrate saturation profiles were measured for glutamate, ammonium and $MnATP^{2-}$. When initial velocity data were plotted according to Eadie-Hofstee or Lineweaver-Burk each substrate gave a single straight line with the adenylylated form of glutamine synthetase. However, with the deadenylylated form of the enzyme a single straight line was obtained only for glutamate, whereas NH_4^+ and $MnATP^{2-}$ gave biphasic saturation curves (data not shown). Table I summarizes the apparent K_m values

Table I. Apparent K_m-values for the Mn^{2+}-dependent biosynthetic activity of the glutamine synthetase from *R. palustris*.

Substrate	K_m (mM)	
	GS-AMP	GS
Glutamate	9.0 ± 1.1	2.0 ± 0.4
Ammonia	0.4 ± 0.1	0.2 ± 0.1
		1.5 ± 0.4
ATP	1.0 ± 0.3	1.2 ± 0.4
		4.8 ± 1.0

When not varied, glutamate was at 100 mM, $MnATP^{2-}$ at 8 mM, and ammonia at 50 mM. The deadenylylated form of glutamine synthetase (GS) was obtained by incubating the adenylylated form (GS-AMP) from an ammonia culture with phosphodiesterase in 10 mM Tris-HCl (pH 7.1) and 1 mM Mn^{2+}, at 35 °C for 60 min. Data are given with standard deviations from at least four experiments.

for the different substrates of glutamine synthetase from *R. palustris*. The data indicate a high affinity of glutamate for the deadenylylated form of the enzyme. The same form of glutamine synthetase, however, showed a high affinity for ammonia and $MnATP^{2-}$ only at low concentrations of substrate and a lower affinity at high substrate concentrations.

Feedback inhibitors

To determine whether feedback inhibition [20] is also a property of the enzyme from *R. palustris*, we investigated the effect of amino acids on the Mn^{2+}-dependent biosynthetic and the transferase activity both of the adenylylated and deadenylylated form of the enzyme. Table II shows that the deadenylylated enzyme species was subjected preferentially to feedback inhibition by alanine, glycine, and serine.

Metabolic interconversion of glutamine synthetase

The adenylylation state of glutamine synthetase of enteric bacteria is considered to reflect the ammonia level in the culture [21]. Glutamine synthetase is largely deadenylylated under ammonia limitation and becomes rapidly adenylylated when ammonia is added to the cells [22]. The extent of inhibition of the transferase activity by Mg^{2+} has been used as a convenient measure to determine the degree of adenylylation of the enzyme, once its isoactivity point is known [17], since Mg^{2+} inhibits the adenylylated form only.

Table II. Feedback inhibition of glutamine synthetase from *R. palustris*.

Effector	Biosynthetic activity % of control		Transferase activity % of control	
	GS	GS-AMP	GS	GS-AMP
None	100	100	100	100
L-Ala	59	73	21	57
Gly	74	89	59	85
L-Ser	62	86	78	89
L-Trp	93	89	100	97
L-His	87	93	97	100
L-Asp	87	97	102	107
L-Gln	90	98	–	–
5′-AMP	96	95	100	99

Effectors were added at 5 mM each. Control activities in the Mn^{2+}-dependent biosynthetic assay for the adenylylated (GS-AMP) and deadenylylated (GS) forms of the enzyme were 2.1 and 1.9 μmol (min · mg protein)$^{-1}$, respectively. Control activities in the transferase assay for the adenylylated and deadenylylated forms were 61.9 and 142.9 μmol (min · mg protein)$^{-1}$, respectively. The deadenylylated glutamine synthetase was obtained from the adenylylated form after treatment with phosphodiesterase.

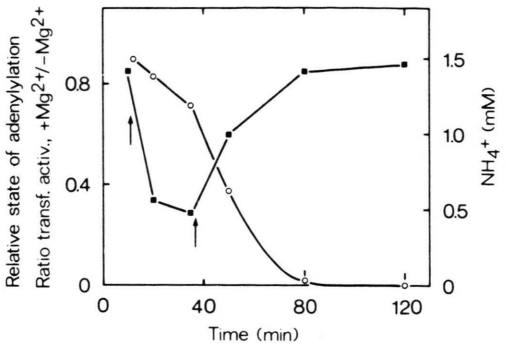

Fig. 5. *In vivo* changes in the adenylylation state of *R. palustris* glutamine synthetase on addition of ammonia. Cells were derived from a culture grown on a limiting amount of yeast extract (0.5 g/l) as only source of nitrogen. A sample of five ml of cell suspension (36.4 mg total protein) in 45 mM phosphate buffer, pH 6.9, was incubated in the light and made 1.5 mM in NH_4Cl (first arrow) and afterwards 18 mM in sodium pyruvate (second arrow). Transferase activity and ammonium concentration (○) were measured as described under Materials and Methods. The relative degree of adenylylation (■) is expressed as the ratio of the transferase activity in the presence and absence of 50 mM Mg^{2+}, measured at pH 6.8.

Glutamine synthetase from *R. palustris* cells grown on malate and a limiting amount of fixed nitrogen, to allow full derepression of nitrogenase, showed only a slight inhibition of the transferase activity by Mg^{2+}. The average ratio of the transferase activity in the presence and absence of Mg^{2+} in these cultures was between 0.8 and 0.9 (1.0 meaning complete deadenylylation). Fig. 5 shows the rapid interconversion of this form of the enzyme to the adenylylated state on addition of 1.5 mM ammonia. Once ammonia was nearly assimilated, supported by the addition of pyruvate to the cell suspension, the degree of adenylylation returned to its original state. In contrast to the nitrogen-limited culture, *R. palustris* grown on 15 mM ammonia had a glutamine synthetase that was strongly inhibited by Mg^{2+} in the transferase assay. The average ratio of $+Mg^{2+}/-Mg^{2+}$ was 0.2–0.3. These results show that the availability of ammonia is an important factor in controlling the regulatory state of the enzyme, and that glutamine synthetase of this organism is subjected to reversible adenylylation *in vivo*.

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[1] K. Alef, H.-J. Burkardt, H.-J. Horstmann, and W. G. Zumft, Z. Naturforsch. **36 c,** 246–254 (1981).
[2] E. R. Stadtman, P. B. Chock, and S. G. Rhee, in: From gene to protein. Information transfer in normal and abnormal cells. Miami Winter Symp., **Vol. 16,** pp. 521–544, (T. R. Russel, K. Brew, H. Faber, and J. Schultz, eds.), Academic Press, New York 1979.
[3] B. C. Johansson and H. Gest, Eur. J. Biochem. **81,** 365–371 (1977).
[4] K. Alef and W. G. Zumft, Abstr. 3rd Internat. Symp. Photosynthetic Prokaryotes, Oxford, (J. M. Nichols, ed.), Dept. Biochem. Univ. Liverpool. B 24 (1979).
[5] B. L. Jones and K. J. Monty, J. Bacteriol. **139,** 1007–1013 (1979).
[6] S. Nordlund and R. Eklund, Abstr. 3rd Internat. Symp. Photosynthetic Prokaryotes, Oxford, (J. M. Nichols, ed.), Dept. Biochem. Univ. Liverpool, B 47 (1979).
[7] W. J. Sweet and R. H. Burris, J. Bacteriol. **145,** 824–831 (1981).
[8] N. Pfennig, Arch. Microbiol. **100,** 197–206 (1974).
[9] G. Kohlhaw, W. Drägert, and H. Holzer, Biochem. Z. **341,** 224–238 (1965).
[10] B. M. Shapiro and E. R. Stadtman, Methods in Enzymology, (H. Tabor, C. White Tabor, eds.), pp. 910–922, Academic Press, New York-London 1970.
[11] B. L. Strehler, in: Methoden der enzymatischen Analyse, 3rd., (H. U. Bergmeyer, ed.), **Vol. 2,** pp. 2165–2172, Verlag Chemie, Weinheim 1974.
[12] J. K. Fawcett and J. E. Scott, J. Clin. Pathol. **13,** 156–159 (1960).
[13] L. H. Stickland, J. Gen. Microbiol. **5,** 696–703 (1951).
[14] B. M. Shapiro, H. S. Kingdon, and E. R. Stadtman, Proc. Nat. Acad. Sci, USA **58,** 642–649 (1967).
[15] R. A. Bender, K. A. Janssen, A. D. Resnik, M. Blumenberg, F. Foor, and B. Magasanik, J. Bacteriol. **129,** 1001–1009 (1977).
[16] J. A. Kleinschmidt and D. Kleiner, Eur. J. Biochem. **89,** 51–60 (1978).
[17] E. R. Stadtman, P. Z. Smyrniotis, J. N. Davis, and M. E. Wittenberger, Anal. Biochem. **95,** 275–285 (1979).
[18] H. S. Kingdon, B. M. Shapiro, and E. R. Stadtman, Proc. Nat. Acad. Sci. USA **58,** 1703–1710 (1967).
[19] E. R. Stadtman, A. Ginsburg, J. E. Ciardi, J. Yeh, S. B. Hennig, and B. M. Shapiro, Adv. Enzyme Regul. **8,** 99–118 (1970).
[20] A. Woolfolk and E. R. Stadtman, Arch. Biochem. Biophys. **118,** 736–755 (1967).
[21] R. M. Wohlhueter, H. Schutt, and H. Holzer, in: The enzymes of glutamine metabolism, (S. Prusiner and E. R. Stadtman, eds.), pp. 45–64, Academic Press New York 1973.
[22] H. Schutt and H. Holzer, Eur. J. Biochem. **26,** 68–72 (1972).