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Multiple Forms of Glutamine Synthetase. Hybrid Formation by Association of Adenylylated and Unadenylylated Subunits†

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ABSTRACT: Fully adenylylated glutamine synthetase ($E_{\overline{12}}$) is rapidly inactivated in the presence of 4 M urea and 0.5 mM ADP, whereas unadenylylated enzyme ($E_{\overline{0.8}}$) is not. The kinetics of urea inactivation of a partially adenylylated enzyme preparation isolated directly from *Escherichia coli* extracts are significantly different from that of an *in vitro* mixture of $E_{\overline{0.8}}$ and $E_{\overline{12}}$ having the same average state of adenylylation. After 30-min exposure to inactivation conditions all adenylylated subunit activity is lost from mixtures of $E_{\overline{0.8}}$ and $E_{\overline{12}}$, but significant adenylylated activity remains in partially adenylylated native enzymes. It is concluded that the native enzyme preparations contain hybrid molecular forms composed of both adenylylated and unadenylylated subunits and that heterologous subunit interactions lead to stabilization of adenylylated subunit activity. Exposure of either $E_{\overline{0.8}}$ or $E_{\overline{12}}$ to 7 M urea at 0° leads to complete dissociation of subunits and complete loss of catalytic activity. Tenfold dilution of the dissociated subunit mixture with Tris·HCl buffer containing KCl, Mg^{2+}, Mn^{2+}, and 2-mercaptoethanol (pH 7.5) results in reassociation of the subunits to produce a 55–65% yield of catalytically active dodecameric aggregates that are indistinguishable from the original enzyme. The yield of active reconstituted enzyme is increased by the presence of ATP or ADP in the reassociation mixture and is diminished by the presence of other substrates and feedback inhibitors of glutamine synthetase including: glutamine, hydroxylamine, potassium arsenate, tryptophan, glycine, CTP, alanine, and AMP. Hybrid dodecameric aggregates produced by reversible dissociation of mixtures of $E_{\overline{12}}$ and $E_{\overline{0.8}}$ are similar to those present in partially adenylylated native enzyme preparations. $E_{\overline{0.8}}$ and $E_{\overline{12}}$ could not be separated from each other by electrophoresis. With succinylation of approximately 36 amino residues per mol wt 600,000, 75% of the catalytic activity is lost but the derivatized enzyme is readily separated from unmodified enzyme by electrophoresis. Hybrids produced by reversible dissociation of a mixture containing equal amounts of $E_{\overline{0.8}}$ and succinylated $E_{\overline{12}}$ are readily separated from $E_{\overline{0.8}}$ and succinylated $E_{\overline{12}}$ by electrophoresis; these hybrids are a mixture of partially adenylylated molecules with from four to nine adenylylated subunits per dodecameric aggregate.

Previous studies have shown that glutamine synthetase from *Escherichia coli* has a mol wt of 600,000 and is composed of 12 apparently identical subunits arranged in two superimposed hexagonal rings (Woolfolk *et al.*, 1966; Shapiro and Ginsburg, 1968; Valentine *et al.*, 1968). The activity is regulated by the covalent attachment of one 5′-adenylyl group to a unique tyrosyl moiety of each subunit (Shapiro *et al.*, 1967; Kingdon *et al.*, 1967). Adenylylation is accompanied by changes in divalent ion specificity, in the pH optimum, and in susceptibility to inhibition by various products of glutamine metabolism and by other effectors (Kingdon *et al.*, 1967; Stadtman *et al.*, 1968). Since each one of the 12 subunits of glutamine synthetase can be adenylylated, the enzyme may exist in multiple molecular forms that differ from each other with respect to the number (0–12) and orientation of adenylylated subunits within single molecules. M. S. Raff and W. C. Blackwelder have calculated that 382 molecular forms of the enzyme are possible (personal communication). Other studies support the conclusion that hybrid forms of the enzyme (*i.e.*, enzyme molecules containing both adenylylated and unadenylylated subunits) do exist and that heterologous interactions between dissimilar subunits affect catalytic parameters (Ginsburg *et al.*, 1970; Denton and Ginsburg, 1970; Segal and Stadtman, 1972) and stability characteristics of the enzyme (Stadtman *et al.*, 1970).

When divalent cations are removed from glutamine synthetase (by treatment with EDTA), it undergoes transition to a "relaxed" state in which tryptophan, tyrosine, and sulfhydryl groups become exposed (Shapiro and Stadtman, 1967; Shapiro and Ginsburg, 1968). On exposure to 1 M urea or pH 8.0 the "relaxed" enzyme is dissociated into mol wt 50,000 inactive subunits (Woolfolk and Stadtman, 1967b; Shapiro and Ginsburg, 1968). In earlier studies it was shown that upon adding divalent cations and decreasing the pH, these subunits reassociated to unstable aggregate forms that were similar but not identical in structure to the native enzyme (Woolfolk and Stadtman, 1967b; Valentine *et al.*, 1968). Reaggregation was accompanied by only transient restoration of catalytic activity.

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The present study was initially concerned with the development of conditions by which reaggregation of dissociated subunits would yield catalytically active enzyme preparations comparable to the native enzyme. After establishing these conditions, hybrid species of enzyme were prepared by the reversible dissociation of mixtures of fully adenylylated ($E_{\overline{12}}$) and unadenylylated ($E_{\overline{0.8}}$) enzymes. Evidence is presented indicating that heterologous interactions between adenylylated and unadenylylated subunits in hybrid enzyme molecules increase the stability of adenylylated subunits to denaturing conditions. Preliminary accounts have been reported (Ciardi *et al.*, 1970; Stadtman *et al.*, 1970; Ciardi and Cimino, 1971).

Materials and Methods

Chemicals. Amino acids, nucleotides, succinic anhydride, tris(hydroxymethyl)aminomethane, and 2,4,6-trinitrobenzenesulfonic acid were obtained from Sigma Chemical Co. and Calbiochem. Urea (Fisher or Merck) was filtered through 0.45-μ Millipore filters and/or recrystallized from 95% ethanol. Imidazole was from Eastman Organic Chemicals, Inc., and 2-methylimidazole and 2,4-dimethylimidazole were from Gallard Schlesinger Co.; solutions were decolorized with activated charcoal before use. All other chemicals were reagent grade.

Incubation Mixtures. (a) ASSAY MIXTURE. Total γ-glutamyltransferase activity was measured as previously described (Kingdon and Stadtman, 1967b) except that the *assay mixture* was modified to contain a pH 7.15 mixed imidazole·HCl buffer (50 mM each of imidazole, 2,4-dimethylimidazole, and 2-methylimidazole), 20 mM glutamine, 20 mM potassium arsenate, 20 mM hydroxylamine·HCl, 0.5 mM ADP, and 0.4 mM Mn^{2+}. To measure the extent of inhibition by various feedback inhibitors the transfer assay was used as previously described (Cimino *et al.*, 1970).

(b) UREA INACTIVATION MIXTURE. Differential inactivation of adenylylated subunits was obtained by incubating the enzyme (0.25 mg/ml) at 37° in the *urea inactivation mixture* containing 187 mM mixed imidazole buffer (pH 7.1), 0.5 mM ADP, 25 mM glutamine, 25 mM potassium arsenate, and 4 M urea. After various periods of time, aliquots were removed and assayed for γ-glutamyltransferase activity and the state of adenylylation.

(c) DISSOCIATION MIXTURE. Complete dissociation of glutamine synthetase subunits was obtained by incubating the enzyme (0.5–1.0 mg/ml) for 1–3 hr at 0°, pH 8.7, in a *dissociation mixture* containing 50 mM Tris·HCl, 1 mM EDTA, 143 mM 2-mercaptoethanol, and 7 M urea. Dissociation was followed by loss of enzyme activity, ultracentrifugation, and electron microscopy.

(d) REASSOCIATION MIXTURE. Reassociation of subunits to form catalytically active dodecameric aggregates was initiated at 0° by a tenfold dilution with a *reassociation mixture* containing 50 mM Tris·HCl (pH 7.5), 0.4 M KCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 72 mM 2-mercaptoethanol. After 30 min, the preparation was removed from the ice bath and allowed to stand at room temperature for several hours. Alternatively the dissociated enzyme preparation was dialyzed overnight at 4° against the reassociation mixture and then slowly allowed to come to room temperature. Reassociation was established by regain of enzyme activity, by ultracentrifugation, and by electron microscopy. Reassociated (reconstituted) enzymes were further dialyzed against 10 mM imidazole·HCl buffer (pH 7.0) containing 1 mM $MnCl_2$ to remove residual urea and to stabilize activity.

State of Adenylylation. The convention adopted by Shapiro and Ginsburg (1968) is used to denote the average state of adenylylation, $E_{\bar{n}}$, of various enzyme preparations. The value of $\bar{n}$ (*i.e.*, the average number of adenylylated subunits per mole of enzyme) may vary from 0 to 12. Thus, unadenylylated enzyme is $E_{\bar{0}}$ and fully adenylylated enzyme is E_{12}, whereas $E_{\overline{7.5}}$ and $E_{\overline{10.4}}$ denote enzyme preparations with 7.5 and 10.4 equiv of adenylylated subunits/mol, respectively. The state of adenylylation was determined enzymatically by measuring the γ-glutamyltransferase activity in the standard assay mixture (see above), which contains 0.4 mM Mn^{2+} as the only divalent cation, and also in the standard assay mixture supplemented with 60 mM Mg^{2+}. Activity in the standard assay mixture is a measure of all subunit activity, whereas activity in the mixture supplemented with 60 mM Mg^{2+} is a measure of unadenylylated subunits only (Stadtman *et al.*, 1970). Therefore, the state of adenylylation is

$$E_{\bar{n}} = 12 - 12\left(\frac{\text{activity in assay mix} + Mg^{2+}}{\text{activity in assay mix}}\right)$$

Alternatively, the state of adenylylation was determined by spectral analysis (Shapiro and Stadtman, 1970b).

Enzyme Preparations. (a) "Natural" enzyme preparations, $E_{\overline{0.8}}$, $E_{\overline{7.5}}$, $E_{\overline{9.0}}$, and $E_{\overline{10.4}}$, were isolated from separate batches of *E. coli* that had been grown under different conditions of nitrogen supply (Holzer *et al.*, 1968; Kingdon and Stadtman, 1967a,b). These enzymes were purified to greater than 95% homogeneity by the method of Woolfolk *et al.* (1966).

(b) "Reconstituted" enzyme preparations were obtained by incubating the enzyme first in the dissociation mixture and then in the reassociation mixture as described above.

(c) "Mixed" enzyme preparations were obtained by mixing $E_{\overline{0.8}}$ and $E_{\overline{12}}$ in various proportions to yield preparations of the desired average states of adenylylation. For example, a preparation containing 56% $E_{0.8}$ and 44% $E_{\overline{12}}$ contains an average of 5.7 adenylylated subunits/mol of enzyme and is referred to as "$E_{5.7}$ mix."

In contrast to *natural* and *reconstituted* enzyme preparations of intermediate states of adenylylation, the *mixed* enzyme preparations do not contain "hybrid" forms (*i.e.*, enzyme molecules composed of both adenylylated and unadenylylated subunits) (Stadtman *et al.*, 1970; Ginsburg *et al.*, 1970; and data presented here).

(d) In other enzyme preparations the E_{12} was prepared by adenylyltransferase catalyzed *in vitro* adenylylation of $E_{\overline{7.5}}$ by the procedure of Ginsburg *et al.* (1970). $E_{\overline{12}}$ containing ^{14}C-labeled adenylyl groups was a gift of Dr. W. B. Anderson. Partially purified adenylyltransferase was a gift of Drs. A. Ginsburg and S. B. Hennig. The $E_{\overline{0.95}}$ was prepared by treating $E_{\overline{7.5}}$ with snake venom phosphodiesterase (Shapiro *et al.*, 1967). Snake venom phosphodiesterase was purchased from Boehringer Mannheim Corp.

Protein. Protein concentrations of the purified glutamine synthetase preparations were estimated from absorbancy measurements at 290 nm (Shapiro, 1969) by a micromodification of the biuret reaction (Layne, 1957) or by the method of Lowry *et al.* (1951).

Succinylation of Adenylylated Glutamine Synthetase. Succinylation (Klotz, 1967) of [^{14}C]adenylyl-labeled glutamine synthetase ($E_{\overline{12}}$) was carried out at pH 7.5 in the presence of a 500 molar excess of succinic anhydride. The succinic anhydride was dissolved in chloroform and added to the reaction vessel.

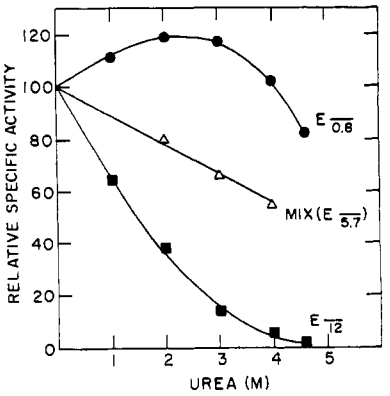

FIGURE 1: Effect of urea concentration on the transferase activity of glutamine synthetase of different states of adenylylation. Activity was determined at pH 7.15 in the modified transfer assay mixture (see Materials and Methods) containing from 0 to 4.6 M urea. The amount of γ-glutamylhydroxamate formed by the enzyme was measured after 15 min at 37°. The enzyme preparations were obtained as described under Materials and Methods.

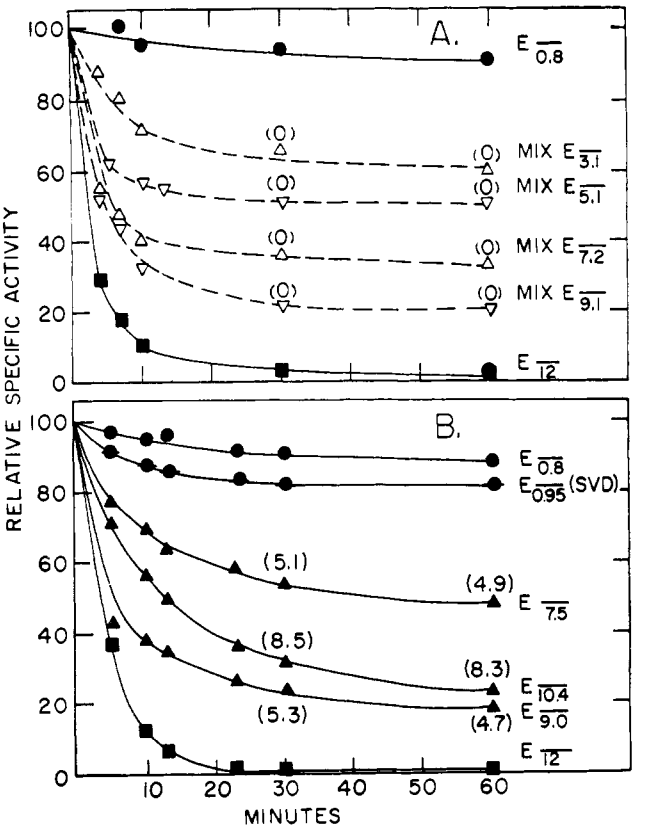

FIGURE 2: Differentiation of glutamine synthetase hybrids from mixtures of adenylylated and unadenylylated enzyme preparations. All enzyme preparations (0.2–0.4 mg/ml), as described under Materials and Methods, were incubated at 37° in the 4 M urea inactivation mixture (Materials and Methods). The enzyme activity and state of adenylylation as measured in the modified transfer assay mix in the presence and absence of Mg^{2+} (Materials and Methods) were determined on aliquots removed after various periods of time. The numbers in parentheses on the curves refer to the state of adenylylation at the time indicated: (A) mixtures of $E_{\overline{0.8}}$ and $E_{\overline{12}}$ (see Materials and Methods); (B) "natural" enzymes.

Just before addition of the enzyme solution, the chloroform was evaporated with a stream of air. The enzyme (1 mg/ml) in 10 mM imidazole·HCl (pH 7.5) and 10 mM $MgCl_2$ was incubated with the succinic anhydride at 4° for 75 min. The succinylated enzyme was then dialyzed against 10 mM imidazole·HCl buffer (pH 7.5) containing 10 mM $MgCl_2$. The number of amino groups modified by succinylation was determined by titrating the unmodified amino groups with trinitrobenzenesulfonic acid (Haynes *et al.*, 1967). Glutamine synthetase (0.67 μM) was incubated for 12 hr with trinitrobenzenesulfonic acid (0.565 mM) in 10 mM imidazole·HCl buffer (pH 7.5) containing 10 mM $MgCl_2$. The number of free amino groups was estimated from the change in absorbancy at 344 nm.

Disc Gel Electrophoresis. Analytical disc gel electrophoresis was carried out with 5% gels at pH 7.2 in bis–Tris·HCl buffer according to the method of Rodbard and Chrambach (1971). Duplicate samples containing about 100 μg of protein were applied to parallel gels; one gel was assayed for γ-glutamyltransferase activity and the other stained with Amido Schwarz to detect protein. In other experiments, after electrophoresis the gels were sliced into 2-mm segments and radioactivity and the state of adenylylation were determined.

Cellulose Acetate Electrophoresis. Cellulose polyacetate strips (Gelman Sephaphore III) were wetted with 50 mM potassium phosphate, pH 6.0, and 2–6 μg of protein were applied. The same buffer was placed in the electrode compartments and a constant 200 V was applied for 1 hr. The protein was then fixed and stained with Ponceau S and Nigrosin as described by Meighen and Schachman (1970).

Isoelectrofocusing. Isoelectrofocusing was carried out on a 110-ml LKB column, with a 0–5% sucrose gradient. A pH gradient of 3–10 was established with 1% ampholyte. A 1-W current was maintained for 48–96 hr until focusing was complete and 1–2-ml samples were collected. The pH, absorbancy at 280 nm, γ-glutamyltransferase activity, and state of adenylylation of each sample were determined.

Sedimentation Coefficients. Sedimentation coefficients were measured with a Spinco Model E ultracentrifuge equipped with an ultraviolet scanning system. The measurements carried out in urea were corrected to the viscosity and density of water at 30° using the equations of Kawahara and Tanford (1966).

Light-Scattering Measurements. Changes in light scattering were measured in the Hitachi (Perkin-Elmer) fluorospectrophotometer at 350 nm. Solutions of 0.5–1.0 mg/ml of protein were filtered five times through a 0.22-μ Millipore filter.

Results

Differential Inactivation of Adenylylated and Unadenylylated Enzymes by the Presence of Urea in the Assay Mixture. Addition of urea (4.0 M) to the standard γ-glutamyltransferase assay mixture leads to inhibition of $E_{\overline{12}}$ but not of $E_{\overline{0.8}}$. As shown in Figure 1, with $E_{\overline{12}}$ there is a nearly linear decline in activity to 15% of the initial value when the concentration of urea added to the assay mixture is increased from 0 to 3 M. Addition of 4–4.5 M urea causes almost complete inhibition. In contrast, little or no inhibition of $E_{\overline{0.8}}$ activity occurs even with 4 M urea; in fact stimulation (up to 20%) of activity is obtained with 2–3 M urea. A mixed enzyme, $E_{\overline{5.7}}$ mix (see Materials and Methods) was inactivated by increasing urea concentration as would be predicted on the assumption that only the $E_{\overline{12}}$ component is affected.

Effect of Urea on the Stability of Hybrid Enzyme Forms. To determine if heterologous interactions between adenylylated and unadenylylated subunits in hybrid molecules affect their stability to inactivation by 4 M urea, the kinetics of inactivation of *mixed* enzyme preparations at various states of adenylylation (see Materials and Methods) were compared with those of *natural* enzyme preparations. As shown in Figure

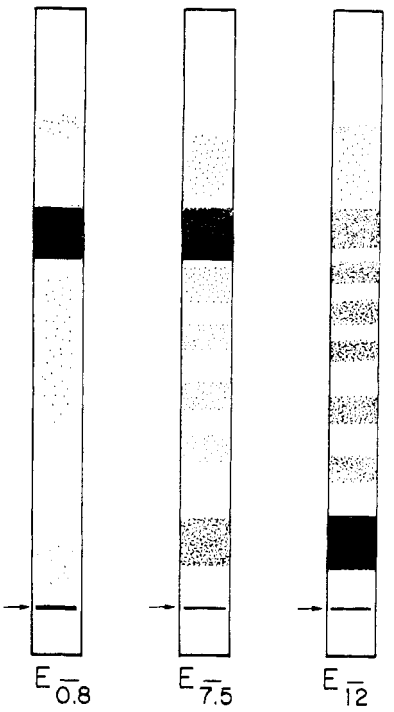

FIGURE 3: Diagramatic representation of polyacrylamide gel separations of enzyme preparations pretreated with 4 M urea. Enzymes (1.5–2.5 mg/ml) were incubated for 15 min at 37° in the urea inactivation mixture (Materials and Methods). An equal volume of 60% sucrose was added and samples (75–100 μg of protein) put on 5% gels at pH 7.2 and current applied for 1 hr (Materials and Methods). Gels were stained with Amido Schwarz to detect protein. The arrows mark the position of the tracking dye at the bottom of each gel. The enzyme preparations wre described in Figure 2. Prior to incubation in the urea inactivation mixture, all preparations exhibited electrophoretic patterns identical with that depicted here for the $E_{\overline{0.8}}$ preparation.

2A, incubation of various mixed enzyme preparations in the urea inactivation mixture led to a rapid partial inactivation; the fraction of enzyme activity ultimately lost was directly proportional to the average number of adenylylated subunits initially present in the mixture. In contrast, as shown in Figure 2B, the natural enzyme preparations were inactivated less rapidly and the extent of inactivation after 10 min was not directly proportional to the average state of adenylylation. Figure 2B also shows that the stability of unadenylylated enzyme ($E_{\overline{0.95}}$), prepared by deadenylylation of $E_{\overline{7.5}}$ with snake venom phosphodiesterase, is about the same as that of $E_{\overline{0.8}}$ enzyme isolated directly from *E. coli*. As is indicated by the numbers on the curves in Figure 2A, no adenylylated subunit activity remained after 30-min exposure of mixed preparations to 4 M urea; all residual activity was due to unadenylylated enzyme. This is as expected from the stability characteristics of the $E_{\overline{0.8}}$ and $E_{\overline{12}}$ preparations (Figure 1) and shows that under these conditions there is probably no interaction between fully adenylylated and unadenylylated enzymes to form hybrid molecules. In contrast, as shown in Figure 2B, when the natural enzyme preparations were exposed to the urea inactivation mixture, the activity that remained after 30 and 60 min was due to both adenylylated and unadenylylated subunits. Thus, the average states of adenylylation of the $E_{\overline{7.5}}$, $E_{\overline{9.0}}$, and and $E_{\overline{10.4}}$ preparations after 60 min were $E_{\overline{4.9}}$, $E_{\overline{4.7}}$, and $E_{\overline{8.3}}$, respectively. Retention of appreciable adenylylated subunit activity after prolonged exposure to conditions that lead to complete inactivation of fully adenylylated enzyme indicates that these partially adenylylated natural preparations contain hybrid forms of enzyme, and that heterologous interactions between adenylylated and unadenylylated subunits within these hybrids protect the adenylylated subunits from denaturation. The fact that residual activity of the $E_{\overline{9.0}}$ preparation exhibited a lower state of adenylylation ($E_{\overline{4.7}}$) than that obtained with $E_{\overline{10.4}}$ suggests that initially the $E_{\overline{9.0}}$ enzyme contained fewer hybrid forms of enzyme, *i.e.*, a higher proportion of fully adenylylated ($E_{\overline{12}}$) molecules.

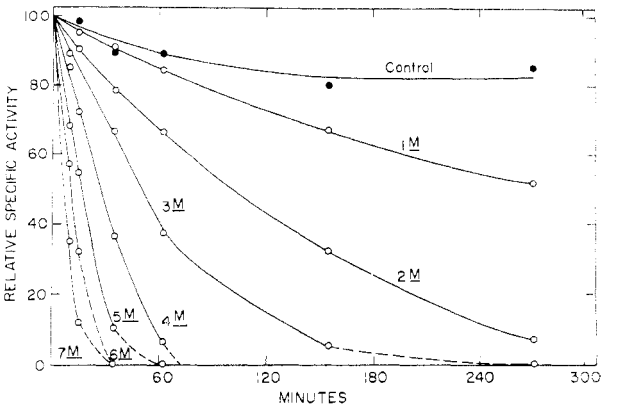

FIGURE 4: Effect of urea concentration on the inactivation of glutamine synthetase. $E_{\overline{12}}$ (0.5 mg/ml) was incubated at 0° at pH 8.7 in a solution containing 50 mM Tris·HCl, 1 mM EDTA, 143 mM 2-mercaptoethanol, and from 1 to 7 M urea. Control was without urea. Aliquots of incubation mixtures were taken at the times shown and assayed for transferase activity (Materials and Methods).

Effect of Urea on the State of Aggregation. Polyacrylamide gel electrophoresis of pure glutamine synthetase preparations at pH 7.2 in the absence of urea yields a single major protein band whose mobility is independent of the state of adenylylation. In 5% gels, the electrophoretic pattern of all preparations ($E_{\overline{0}}$ to $E_{\overline{12}}$) is identical with that depicted in Figure 3 for the $E_{\overline{0.8}}$ following its prior incubation for 15 min in the urea inactivation mixture (Figure 3). However, electrophoresis of $E_{\overline{12}}$ that had been exposed to the inactivation mixture for 15 min resulted in the separation of multiple bands, the most prominent of which was the fastest band corresponding in mobility to a subunit species of 50,000 mol wt. The urea treated $E_{\overline{7.5}}$ preparation exhibited a similar spectrum of protein bands but the slowest band, corresponding to the undissociated enzyme, was present in greatest amount and was the only protein band with demonstratable catalytic activity. Parallel experiments showed that at 25° urea treatment provokes a marked decrease in light-scattering ratio of $E_{\overline{12}}$ but not of $E_{\overline{0.8}}$.

The electrophoretic patterns obtained following treatment with urea are not affected by omission of hydroxylamine from the prior incubation medium; however, with omission of either ADP or Mn^{2+}, all forms of the enzyme (including $E_{\overline{0.8}}$) are inactivated and in the case of ADP omission (Mn^{2+} omission not tested) yield complex electrophoretic patterns similar to those shown for $E_{\overline{12}}$ in Figure 3. It therefore appears that the difference in susceptibility of unadenylylated and adenylylated enzyme forms to dissociation by 4 M urea is related to differences in the capacity of ADP and Mn^{2+} to protect the enzymes against such denaturation.

Effect of Urea Concentration on Inactivation (Dissociation) of Glutamine Synthetase. Figure 4 shows the effect of urea concentration on the inactivation of glutamine synthetase ($E_{\overline{12}}$) in the presence of 50 mM Tris·HCl (pH 8.7), 143 mM 2-mercaptoethanol, and 1 mM EDTA. Inactivation of $E_{\overline{12}}$ at all urea concentrations studied (1–7 M) appears to be a pseudo-

unimolecular process with respect to enzyme since there is a nearly linear relationship between the logarithm of the catalytic activity and time. The half-lives vary from 300 min with 1 M urea to 5 min with 7 M urea.

Other studies show that $E_{\overline{0.8}}$, $E_{\overline{7.5}}$, and $E_{\overline{12}}$ exhibit similar inactivation kinetics in the presence of high urea concentration (4–7 M). With lower urea concentrations the rates of inactivation are dependent upon the state of adenylylation (Figure 2).

The remarkable stability of glutamine synthetase in the presence of 1 mM EDTA at pH 8.7, in the absence of added urea (upper curve, Figure 4), was unexpected since relaxed enzyme (obtained by prior treatment at pH 7.0 with EDTA) undergoes rapid dissociation at pH 8.0 and above (Woolfolk and Stadtman, 1967b). This apparent discrepancy was explained by the discovery that addition of EDTA to enzyme at pH 8.7 does not rapidly produce the unstable configuration (*i.e.*, relaxed enzyme), whereas incubation of the enzyme with 1 mM EDTA at pH 7.0 prior to adjustment of the pH to 8.7 leads to rapid, complete inactivation of both $E_{\overline{0.8}}$ and $E_{\overline{12}}$, even in the absence of urea. In the experiment shown in Figure 4, the enzyme was added last to the alkaline incubation mixture (pH 8.7) containing 1 mM EDTA.

Effect of Dissociation Conditions on Recovery of Activity upon Reassociation. In earlier studies, Woolfolk and Stadtman (1967b) showed that, in the presence of 1.5 M urea and EDTA at pH 8.0, inactivation of glutamine synthetase is accompanied by complete dissociation of the enzyme subunits and that reassociation of these subunits to highly unstable aggregates could be provoked by addition of Mn^{2+} and lowering of the pH to 7.5 (1.5 M urea still present). Reaggregation under these conditions leads only to transient recovery of up to 60% of the initial catalytic activity.

In the present study it was found that catalytically stable dodecameric aggregates very similar, if not identical, to those of the native enzyme can be obtained upon reassociation if dissociation is carried out in the standard dissociation mixture (see Materials and Methods). When subunits prepared under these conditions are exposed to the standard reassociation conditions (see Materials and Methods), there is a 55–65% recovery of catalytically active dodecameric aggregates that are indistinguishable from the native enzyme. The presence of 1 mM EDTA or 5% sucrose in the dissociation mixture did not affect dissociation of enzyme nor the recovery of activity during subsequent reassociation. However, omission of 2-mercaptoethanol during dissociation led to lower recoveries (30%) of active enzyme. The time of exposure of the enzyme to the dissociating conditions could be varied from 1 to 24 hr without affecting the yield of enzyme activity upon reassociation; however, exposure to dissociating conditions for 7 days (0–4°) resulted in variable recoveries (15–40%) of initial enzyme activity. Although dissociation by 7 M urea occurs at all pH values studied over the range of 7.2–8.9, maximal recovery of activity was obtained when dissociation was at pH 8.7.

Reassociation of Subunits. When divalent cations are omitted from the reassociation mixture there is almost no reaggregation of subunits to form active enzyme; moreover, KCl and probably 2-mercaptoethanol are necessary for maximum restoration of activity.

Of the divalent cations tested, Mn^{2+}, Mg^{2+}, Ba^{2+}, and Ca^{2+} can all support reactivation of the dissociated enzyme. Of these Mn^{2+} is the most effective. At 9 mM concentration recovery of activity with Mg^{2+} is 50% as great as with Mn^{2+} and with $CaCl_2$ or $BaCl_2$ (35%); however, at a higher concentration (50 mM), Mg^{2+} is as effective as 9 mM Mn^{2+}. Other cations tested including Zn^{2+}, Cd^{2+}, Hg^{2+}, and Ni^{2+} (5–10 mM) did not promote recovery of enzyme activity, but they inhibited the recovery obtained with Mg^{2+}. Substitution of LiCl for KCl resulted in 20% less recovery of activity. The pH optimum for reactivation is between pH 7.5 and 8.0 with either Tris or imidazole buffer. Lower recovery of catalytic activity occurs in potassium phosphate buffers (50 mM), which however show a broader pH optimum range (7.5–9.0). The extent of reassociation is both time and temperature dependent. For best results reassociation is initiated at 0° for at least 30 min. Following this initial treatment up to 65% of the enzyme activity can be recovered by either allowing the sample to slowly warm and remain at 25° for several hours or leaving it overnight at 4° and then bringing to room temperature (see Table I and Figure 5).

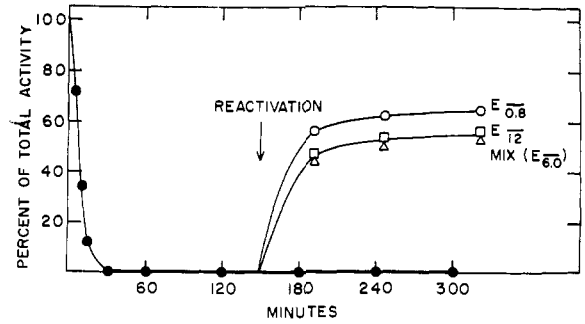

FIGURE 5: The time course of inactivation and reactivation of glutamine synthetase under conditions for dissociation and reassociation. Enzyme preparations (0.5 mg/ml) of $E_{\overline{0.8}}$, $E_{\overline{12}}$, and a mixture of both, $E_{\overline{6.0}}$ mix, were subjected to standard dissociation and reassociation conditions as described under Materials and Methods. Enzyme activity was measured at 37° by the modified transfer assay on aliquots removed at various times.

Time Course of Inactivation and Reactivation. As shown in Figure 5, when either $E_{\overline{0.8}}$, $E_{\overline{12}}$, or a mixture of both to yield $E_{\overline{6.0}}$ is exposed to standard dissociating conditions, complete loss of activity is observed in 30 min. With tenfold dilution in reassociating medium, enzyme activity is rapidly restored

TABLE I: Requirements for the Reassociation of Glutamine Synthetase.[a]

Omission	% of Initial Act. Recovered after Complete Inactivation	
	2 hr	20 hr
None	55	64
KCl	34	41
2-Mercaptoethanol	52	60
$MnCl_2$	22	34
$MgCl_2$	53	64
$MnCl_2$ and $MgCl_2$	<2	<2

[a] The enzyme was a mixture of $E_{\overline{0.8}}$ and $E_{\overline{12}}$ (0.5 mg/ml) with an average state of adenylylation of $E_{\overline{7.1}}$. Activity was measured by the modified γ-glutamyltransferase assay. The enzyme mixture was first completely inactivated (dissociated) by incubation in the dissociation mixture as described under Materials and Methods. Reassociation was achieved by tenfold dilution of the dissociated subunits with the reassociation mixture (see Materials and Methods) which was modified as indicated.

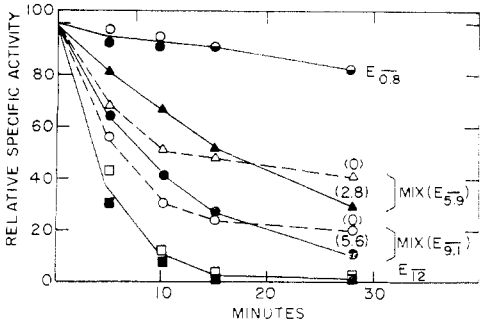

FIGURE 6: Properties of hybrids formed *in vitro* by dissociation and reassociation of mixtures of E_{12} and $E_{0.8}$. Enzyme preparations (0.5 mg/ml) of $E_{0.8}$, E_{12}, and two mixtures of them, $E_{5.9}$ mix and $E_{9.1}$ mix, were completely dissociated and reassociated as described under Materials and Methods. The reconstituted (reassociated) enzymes are represented by closed symbols. The open symbols are undissociated controls. Both reconstituted and control enzymes (0.02 mg/ml) were incubated at 37° in the urea inactivation mixture (Materials and Methods). The enzyme activity and state of adenylylation were measured as described in Figure 2. The numbers in parentheses on the curves refer to the state of adenylylation at the time indicated.

to approximately 55–65% of the original value within a few hours. The activity of reassociated enzyme prepared in this manner declines slowly after about 24 hr, possibly due to the 0.7 M residual urea from the dissociation medium. However, if the reconstituted enzyme is extensively dialyzed against 10 mM imidazole·HCl buffer containing 1 mM $MnCl_2$ (pH 7.0), its activity is stable for more than 6 months.

During incubation in the standard dissociating mixture, glutamine synthetase is converted from a homogeneous protein having a sedimentation coefficient $s_{20,w} = 20$ S to a slow sedimenting species of $s_{20,w} = 2.2$ S corresponding to the mol wt 50,000 subunit. Dialysis of the dissociated sample against reassociation mixture yields active enzyme with a sedimentation coefficient of $s_{20,w} = 20.1$ S. The product of reaggregation is therefore similar if not identical in size to native glutamine synthetase. E. Shelton has shown (personal communication) that, after dissociation, no intact or partially degraded molecules can be observed with the electron microscope; however, following reassociation to active enzyme, electron micrographs show the presence of uniform double hexagonal structures characteristic of the native enzyme (Valentine *et al.*, 1968). Direct comparisons of kinetic parameters including divalent ion specificity, inhibitability by feedback effectors, stability to denaturing conditions, and pH optima did not disclose significant differences between native enzyme and reconstituted enzymes obtained by subunit dissociation and reassociation.

Effect of Feedback Inhibitors and Substrates on Reassociation. Addition of ATP (1 mM) or ADP (0.4 mM) to the reassociation mixture resulted in a slight increase in recovery of transferase activity, from 58% in the absence of effectors to 66% in the presence of effectors (Table II). Other substrates and cofactors for the transferase reaction at concentrations of 18–20 mM inhibited reassociation; the order of effectiveness in promoting reassociation was glutamine < hydroxylamine < potassium arsenate. The three allosteric inhibitors, tryptophan, glycine, and CTP, which are competitive with respect to the substrate, glutamate (Woolfolk and Stadtman, 1967a), also inhibited reassociation; glycine and CTP, the most effective inhibitors tested, allowed only 4–5% recovery of enzyme activity. Histidine (18 mM) which is a competitive inhibitor of the substrate, NH_3, does not affect reassociation, whereas the noncompetitive inhibitors, alanine and AMP (Woolfolk and Stadtman, 1967a), cause partial inhibition of reassociation.

TABLE II: Effect of Feedback Inhibitors and Substrates on Subunit Reassociation of Glutamine Synthetase.[a]

Effector	Concn (mM)	Transferase Act. of Reconstituted Enzyme[b]
None		58
Adenosine triphosphate	1.0	67
Adenosine diphosphate	0.4	65
Glutamine	20	47
Hydroxylamine	20	45
Potassium arsenate	20	23
Tryptophan	18	33
Glycine	18	4
Cytidine triphosphate	18	5
Histidine	18	61
Adenosine monophosphate	18	52
Alanine	18	31

[a] $E_{\overline{0.8}}$ (0.25 mg/ml) was used for the test with adenosine triphosphate. For all other tests the enzyme was a mixture of $E_{0.8}$ and E_{12} (0.43 mg/ml, $E_{8.0}$ mix). The enzymes were first incubated in the dissociation mixture and then reconstituted by incubation in a standard reassociation mixture (see Materials and Methods) that was supplemented with various effectors as indicated. [b] The transferase activity of the reconstituted enzyme refers to the percentage of γ-glutamyltransferase activity exhibited by the original undissociated enzyme preparation as measured in the standard assay mixture.

Formation of Hybrids by Dissociation and Reassociation of Mixtures of E_{12} and $E_{0.8}$. Figure 6 shows the kinetics of urea inactivation of original preparations of $E_{\overline{0.8}}$, $E_{\overline{12}}$, $E_{5.9}$ mix, and $E_{9.1}$ mix, and of the reconstituted enzyme preparations derived from them by their exposure first to the subunit dissociation mixture and then to the reassociation mixture. Neither the original nor the reconstituted preparation of $E_{0.8}$ is inactivated by exposure to the urea inactivation mixture, whereas the original and reconstituted preparations of $E_{\overline{12}}$ are both rapidly inactivated completely at identical rates. These results are further evidence that reconstituted enzymes arising from the reassociation of homologous subunits (either adenylylated or unadenylylated subunits) are indistinguishable from their naturally occurring counterparts. On the contrary, the kinetics of inactivation of the original and reconstituted mixed enzymes preparations are distinctly different. Exposure of the original mixed enzymes to urea inactivation mixture led to rapid loss of only that fraction of the initial activity due to adenylylated subunits (50% for the $E_{\overline{5.9}}$ mix and 75% for the $E_{9.1}$ mix). After 30 min the average state of adenylylation of both preparations was $E_{\overline{0}}$. In contrast, the *reconstituted* $E_{\overline{5.9}}$ mix and $E_{\overline{9.1}}$ mix preparations were inactivated by urea more slowly than the original mixtures from which they were derived and after 30 min the average state of adenylylation of the residual activity was $E_{\overline{2.8}}$ for the $E_{\overline{5.9}}$ mix and $E_{\overline{5.6}}$ for the $E_{\overline{9.1}}$ mix. These results indicate that reassociation of mixtures of unadenylylated and adenylylated subunits gives rise to hybrid enzyme forms similar to naturally occurring enzymes of the same intermediate states of adenylylation (*cf.* Figure 2B).

TABLE III: Influence of Hybridization on Effector Responses of Glutamine Synthetase.[a]

Effector	Relative Transferase Activity				
	$E_{\overline{0.8}}$	$E_{\overline{12}}$	$E_{\overline{6.8}}$ Mix	Reconstituted Hybrids[b] ($E_{\overline{7.0}}$)	Natural Hybrid ($E_{\overline{7.5}}$)
None	100	100	100	100	100
Alanine	5	19	16	18	17
Glycine	42	57	55	50	55
Histidine	110	67	78	83	81
Tryptophan	97	50	68	63	61
AMP	116	26	60	58	50
CTP	118	19	52	54	46

[a] The γ-glutamyltransferase assay was used (see Materials and Methods). Effectors were present at 20 mM concentration. [b] The reconstituted hybrids were formed by dissociation and reassociation of the $E_{\overline{6.8}}$ mix preparation as described under Materials and Methods. Other enzyme preparations are described under Materials and Methods.

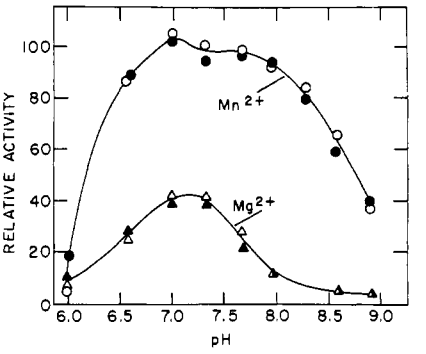

FIGURE 7: A comparison of the pH–activity profiles of a reconstituted hybrid enzyme preparation and the $E_{\overline{7.1}}$ mix from which it was prepared. The γ-glutamyltransferase activity was measured in the presence of mixed imidazole buffer and 0.4 mM Mn^{2+} as described under Materials and Methods. Where indicated, 60 mM Mg^{2+} was also added. The open symbols refer to the $E_{\overline{7.1}}$ mix enzyme preparation. The closed symbols refer to the reconstituted hybrid enzyme preparations prepared by incubating the $E_{\overline{7.1}}$ mix first with the dissociation mixture and then with the reassociation mixture (Materials and Methods). Approximately 65% of the activity measured before dissociation was recovered in the reconstituted enzyme preparations with both the Mn^{2+} and Mg^{2+} + Mn^{2+} assays and these measurements were normalized with respect to the $E_{\overline{7.1}}$ mix at pH 7.15.

In another experiment, a natural hybrid, $E_{\overline{7.5}}$, isolated directly from *E. coli*, and a reconstituted enzyme, derived from it by dissociation and reassociation, were both exposed to the 4 M urea inactivation conditions. The rate of inactivation during the first 10 min was greater for the reconstituted enzyme than for the original enzyme (34 *vs.* 18%) but, from 10 to 60 min, rates were nearly the same; after 30 min the measured states of adenylylation were $E_{\overline{4.9}}$ and $E_{\overline{5.5}}$, respectively.

Properties of Reconstituted Hybrid Enzyme Forms. Earlier studies (Stadtman *et al.*, 1968) showed that the γ-glutamyltransferase activities of $E_{\overline{12}}$ and $E_{\overline{0.8}}$ differ significantly with respect to pH optimum, divalent cation specificity, and inhibitability by end products of glutamine metabolism. In the presence of Mn^{2+} the pH optima for $E_{\overline{12}}$ and $E_{\overline{0.8}}$ are 6.9 and 8.1, respectively, whereas, in the presence of Mg^{2+}, $E_{\overline{12}}$ has no activity and the pH optimum of $E_{\overline{0.8}}$ is shifted to 7.1. Moreover, the pH–activity profiles of mixtures of $E_{\overline{0.8}}$ and $E_{\overline{12}}$ in the presence of either divalent cation reflect exactly the proportions of the two enzyme forms in the mixture. Figure 7 shows that the pH–activity profile of a reconstituted hybrid enzyme preparation ($E_{\overline{7.1}}$) in the presence of either Mg^{2+} or Mn^{2+} is identical with that for the $E_{\overline{7.1}}$ mix from which it was derived.

Other data summarized in Table III show that the γ-glutamyltransferase activities of a reconstituted $E_{\overline{7.1}}$ hybrid and the $E_{\overline{6.8}}$ mix from which it was derived and a natural enzyme preparation ($E_{\overline{7.5}}$) are all equally sensitive to inhibition by various end products of glutamine metabolism.

Results of these studies and those reported in Figure 7 are similar to results obtained with hybrid enzyme preparations produced by controlled phosphodiesterase digestion of $E_{\overline{12}}$ (Stadtman *et al.*, 1968, 1970). The data indicate that the γ-glutamyltransferase activities of adenylylated and unadenylylated subunits within hybrid enzyme molecules are expressed independently and are not influenced by heterologous interaction between subunits.

Hybridization of Native and Succinylated Glutamine Synthetase. Adenylylation of a single tyrosyl hydroxyl group of a protein should result in a unit increase in the net negative charge of the protein. Accordingly, attachment of from 0 to 12 adenylyl groups to a molecule of glutamine synthetase should yield up to 13 different variants that differ from each other with respect to their net negative charge. Nevertheless, it has not been possible to separate $E_{\overline{12}}$ from $E_{\overline{0.8}}$ by electrophoresis or by electrofocusing. The isoelectric point of both enzyme forms is 4.9.

To obtain a preparation of adenylylated enzyme that could be separated from unadenylylated enzyme by electrophoresis, the $E_{\overline{12}}$ was succinylated by the procedure of Meighen and Schachman (1970). Conditions were selected that led to succinylation of an average of 36 amino groups per 600,000 mol wt (*i.e.*, 3 per subunit), as judged by the loss in capacity to undergo trinitrophenylation (Haynes *et al.*, 1967). Succinylation was accompanied by a loss of 75% of γ-glutamyltransferase activity but the pH optimum, divalent ion specificity, and inhibitability by feedback effectors of the residual activity were the same as for unmodified enzyme. The electrophoretic mobility of the succinylated $E_{\overline{12}}$ was significantly different from that of unmodified $E_{\overline{0}}$ or $E_{\overline{12}}$ and it was easily separated from these by electrophoresis in polyacrylamide gel or on cellulose acetate. To determine if the succinylated enzyme would undergo reversible dissociation and form hybrids with unmodified $E_{\overline{0}}$ subunits, the succinylated $E_{\overline{12}}$, unmodified $E_{\overline{0}}$, and a mixture containing equal amounts of both were each incubated first in the dissociation mixture and then in the reassociation mixture as described under Materials and Methods. Figure 8 shows that the reconstituted $E_{\overline{0.8}}$ and succinylated $E_{\overline{12}}$ preparations are readily separated from one another by electrophoresis on cellulose acetate (*cf.* strips 1, 2, and 4, Figure 8). However, the reconstituted enzyme derived from a mixture of $E_{\overline{0.8}}$ and succinylated $E_{\overline{12}}$ moves as a rather diffuse band of intermediate electrophoretic mobility, and is readily separated from the parental types (see strips 3 and 5, Figure 8). Similar separations are obtained during electrophoresis on polyacrylamide gel. These results demonstrate the production of electrophoretically separable variants by reaggregation of mixtures of unadenylylated and succinylated–adenylylated subunits and offer direct proof for the existence of hybrid species of glutamine synthetase. The diffuseness of the electrophoretic band of the reconstituted enzyme compared to

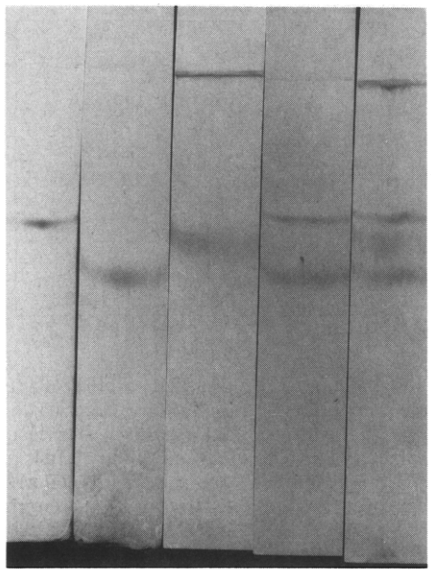

FIGURE 8: Separation of reconstituted hybrid glutamine synthetases from the reconstituted succinylated–adenylylated ($E_{\overline{12}}$) and unadenylylated ($E_{\overline{0.8}}$) enzymes from which they were prepared. Succinylated $E_{\overline{12}}$, unmodified $E_{\overline{0.8}}$, and a mixture containing equal amounts of both were each subjected to dissociation and reassociation conditions (Materials and Methods). Two to six micrograms of protein were applied to cellulose acetate strips and electrophoresis carried out as described under Materials and Methods. Protein was stained with Ponceau S and Nigrosin. The bands of the strips, from left to right, are: (1) $E_{\overline{0.8}}$, (2) succinylated $E_{\overline{12}}$, (3) partially succinylated hybrids, (4) $E_{\overline{0.8}}$ and succinylated $E_{\overline{12}}$, and (5) all three glutamine synthetase preparations.

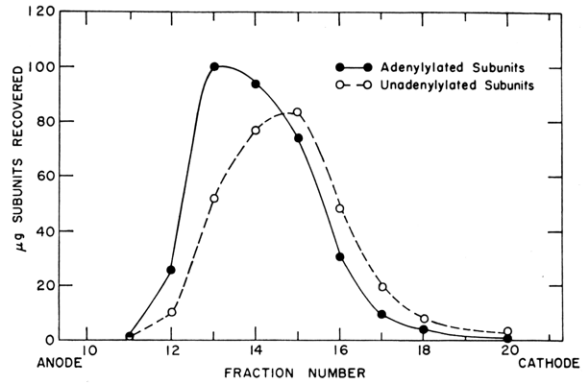

FIGURE 9: Distribution of succinylated–adenylylated and unadenylylated subunits in reconstituted hybrid enzymes following reversible dissociation. Succinylated [^{14}C]adenylyl-$E_{\overline{12}}$ (with 25% residual transferase activity) was mixed with an equal amount of $E_{\overline{0.8}}$. The mixed enzymes were dissociated into subunits and the subunits subsequently reassociated as described under Materials and Methods. The resultant reconstituted enzyme mixture was subjected to electrophoresis on an acrylamide disc gel at pH 7.2 (Materials and Methods). The gel was then sliced into 1-mm sections and the enzyme eluted with 10 mM imidazole·HCl–1 mM $MnCl_2$, pH 7.0. Aliquots of each fraction were assayed for Mg^{2+}-dependent γ-glutamylhydroxamate activity as a measure of unadenylylated subunits and radioactivity was measured by liquid scintillation to estimate adenylylated subunits. The amount of each type of subunit was calculated for each fraction.

either the $E_{\overline{0}}$ or succinylated $E_{\overline{12}}$ preparation suggests that the reconstituted enzyme is a mixture of hybrid forms that differ from each other in electrophoretic mobility. To determine if this is the case, hybrids were produced by dissociation and reassociation of a mixture of $E_{\overline{0.8}}$ and succinylated ^{14}C-adenylylated $E_{\overline{12}}$. The reconstituted ^{14}C-labeled enzyme was then subjected to electrophoresis on polyacrylamide gel and the diffuse protein band thus obtained was fractionated and each fraction assayed for ^{14}C (as a measure of adenylylated subunits) and for unadenylylated subunits by measuring transferase activity in the presence of 60 mM Mg^{2+}. Figure 9 shows that, progressing from the anode end to the cathode end of the gel, both activities are contained in fractions 12–18. As would be expected for a heterogenous mixture of hybrids, there is a higher proportion of ^{14}C-adenylylated–succinylated subunits in that portion of the protein band closest to the anode (fractions 12–14) and a higher proportion of unadenylylated subunits in the end closest to the cathode (fractions 15–18). From the distribution of both activities it could be calculated that the average state of adenylylation varied from $E_{\overline{9.0}}$ to $E_{\overline{4.0}}$ in fractions 12–17. This is in good agreement with direct determination of the state of adenylylation as measured by the transferase activities in Mn^{2+} and Mg^{2+} (see Materials and Methods) which varied from $E_{\overline{8.0}}$ to $E_{\overline{4.0}}$ over the same range of fractions.

Discussion

Previous studies showed that adenylylation of glutamine synthetase produces marked changes in the catalytic potential, pH optimum, divalent cation specificity, and inhibitability to some feedback effectors (Kingdon *et al.*, 1967; Stadtman *et al.*, 1968, 1970). The present study shows that adenylylation also affects the ability of Mn^{2+} and ADP to protect the enzyme from inactivation and dissociation by 4 M urea. The capacity of Mn^{2+} (or Mg^{2+}) alone to produce a more stable, tightened configuration of glutamine synthetase is well established (Kingdon *et al.*, 1968; Shapiro and Ginsburg, 1968). Whereas the tightening by divalent cations is not of itself sufficient to protect the enzyme from inactivation by urea, the combined action of Mn^{2+} and ADP does protect the unadenylylated, but not the adenylylated, enzyme from inactivation. This implies that the binding of ADP to unadenylylated enzyme tightened with Mn^{2+} provokes a further conformational change that lends greater stability to the subunit structure and strengthens the interactions between subunits.

The capacity of Mn^{2+} and ADP to protect unadenylylated but not adenylylated enzyme may be related to the large difference in the affinities of these ligands for the two enzyme forms. The affinity of Mn^{2+} for unadenylylated enzyme is about 100 times greater than for adenylylated enzyme (Denton and Ginsburg, 1969), and the apparent affinity of ADP ($1/K_m = 2 \times 10^7$ M) for unadenylylated enzyme is about 2000 times its affinity for adenylylated enzyme ($1/K_m = 1.2 \times 10^4$ M) (P. Smyrniotis and E. R. Stadtman, unpublished data). Whereas the concentrations of Mn^{2+} and ADP used in the present studies were sufficient to saturate both enzyme forms under normal assay conditions, in the presence of 4 M urea the affinity of one or both ligands may be depressed such that only the unadenylylated enzyme is saturated.

Wilk *et al.* (1969) have shown that ADP (or ATP) and Mn^{2+} (or Mg^{2+}) also protect the sheep brain glutamine synthetase from urea inactivation and dissociation and Tiemeier and Millman (1972) reported that ATP, acting at the catalytic site, protects Chinese hamster liver glutamine synthetase from inactivation by heat or *N*-ethylmaleimide. There is no evidence that the mammalian enzymes can be adenylylated.

At alkaline pH (>8.0) and in the absence of Mn^{2+} and ADP, all forms of glutamine synthetase are rapidly dissociated to subunits by concentrations of urea of 4 M or greater. A minimum concentration of 4 M urea is required also for the complete dissociation of pyruvate kinase (Steinmetz and Deal,

1966), aldolase (Stellwagen and Schachman, 1962), and glyceraldehyde-3-phosphate dehydrogenase (Deal, 1963).

Although complete dissociation of glutamine synthetase subunits is achieved by 4 M urea (pH 8.7, 0°), the integrity of the subunit species (as judged by their capacity to reassociate to form active enzyme) is not as great as for subunits produced in the presence of 7 M urea. After dissociation of the enzyme at pH 8.7 in the presence of 7 M urea and 2-mercaptoethanol, best recovery of enzyme activity is obtained by tenfold dilution at 0° with a solution containing divalent cation and KCl, pH 7.5, followed by a slow increase in temperature to 25°. The effects of temperature suggest that disruption of critical hydrophobic bonds at 0° and their orderly regeneration with a slow increase in temperature play an important role in the recovery of enzyme (Kauzmann, 1959).

For maximal recovery of catalytic activity in the reconstituted enzyme, a mercaptan (2-mercaptoethanol) must be present during the dissociation and reassociation of subunits. Presumably the sulfhydryl compound prevents oxidation of enzyme sulfhydryl groups, permitting the subunits to assume their native configuration. It is not known whether the presence of divalent cations is needed to stabilize a conformation of the dissociated subunits that favors its reassociation, or if it is necessary only to tighten the dodecameric aggregate once it is formed. Perhaps both effects are involved. High ionic strength (KCl) possibly aids in the overall process by preventing undesirable ionic interactions. Whether or not ADP might replace KCl and/or divalent cations under these conditions was not tested. The addition of ADP or ATP to reactivation mixtures containing KCl and divalent cations does lead to a higher yield of reconstituted enzyme activity; however, when dissociated subunits are diluted into the transfer assay mixture which contains both ADP and Mn^{2+}, reactivation does not occur.

The conditions for subunit dissociation and/or reassociation used here are similar to those used in studies with pyruvate kinase (Johnson *et al.*, 1969), glyceraldehyde-3-phosphate dehydrogenase (Deal, 1969; Stancel and Deal, 1969), and glyoxylic acid reductase (Kohn, 1970).

Under the conditions used for subunit dissociation and reassociation, the average state of adenylylation of the reconstituted enzyme formed is about the same as that of the original enzyme preparation; moreover, the yield of active enzyme is always about 55–65%. Most of the inactive protein appears as large aggregates that either precipitate from solution or fail to move during electrophoresis on polyacrylamide gels or cellulose acetate. Reassociation thus leads to the formation of a constant fraction of catalytically inactive aggregates of random subunit composition.

The existence of enzyme molecules (hybrids) containing both adenylylated and unadenylylated subunits and the fact that heterologous interactions occur between dissimilar subunits in these hybrid molecules were deduced from earlier studies showing that for each kind of subunit the specific activity and the apparent K_m for substrates varied with the average state of adenylylation (Ginsburg *et al.*, 1970; Segal and Stadtman, 1972). The existence of hybrid molecules is supported further by the present results which show that kinetics and extent of enzyme inactivation at pH 7.1, in the presence of 4 M urea, 0.4 mM Mn^{2+}, and 0.5 M ADP, vary with the average state of adenylylation. Surely, the fact that appreciable adenylylated subunit activity of partially adenylylated enzyme survives exposure to conditions that completely inactivate fully adenylylated enzyme is proof that hybrid molecules exist and that heterologous interactions between the adenylylated and unadenylylated subunits in these hybrids stabilize the adenylylated subunit.

The increased stability of adenylylated subunits disclosed the presence of a high proportion of hybrid molecules in reconstituted enzyme preparation obtained when mixtures of fully adenylylated and unadenylylated enzyme were subjected to dissociation and reassociation conditions. From a comparison of preparations having the same average state of adenylylation, it appears that synthetic hybrids obtained from a mixture of $E_{\overline{0.8}}$ and $E_{\overline{12}}$ are similar but not identical with the native hybrids isolated directly from *E. coli*. Moreover, when a natural enzyme preparation, $E_{\overline{7.5}}$, was subjected to complete dissociation and reassociation of its subunits, the reconstituted enzyme, also $E_{\overline{7.5}}$, contained a higher percentage of more labile species than did the undissociated $E_{\overline{7.5}}$ from which it was derived. Earlier studies (Stadtman *et al.*, 1970; Ginsburg *et al.*, 1970) indicated a difference in the behavior of hybrids derived from limited treatment of $E_{\overline{12}}$ with snake venom phosphodiesterase and those produced during growth or by *in vitro* adenylylation of $E_{\overline{2.3}}$. Segal and Stadtman (1972) presented evidence suggesting that adenylylation of a subunit of glutamine synthetase leads not only to inactivation of its Co^{2+}-supported biosynthetic activity but also to inactivation of those unadenylylated subunits adjacent to it. Therefore, the differences in stability of natural and synthetic hybrids could reflect either differences in the proportions of highly adenylylated and relatively unadenylylated enzyme molecules or differences in the distribution of adenylylated and unadenylylated subunits within hybrid molecules.

In any case, subtle differences between natural hybrids and synthetic hybrids are not surprising since at least 384 different molecular forms of the enzyme are possible (M. S. Raff and W. C. Blackwelder, personal communication; Segal and Stadtman, 1972). The *in vitro* synthesis of molecular hybrids from mixtures of adenylylated and unadenylylated subunits appears to be a purely random process (as judged by the composition of hybrids derived from succinylylated–adenylylated and unadenylylated subunits (Figure 9)). However, *in vivo* synthesis of hybrids may not be completely random since it involves the covalent attachment to and removal of adenylyl groups from subunits of the dodecameric enzyme, and this is catalyzed by a multicomponent enzyme system that is rigorously controlled by the intracellular concentrations of various metabolites, *e.g.*, UTP, ATP, P_i, glutamine, α-ketoglutarate, and others (Shapiro and Stadtman, 1970a; Brown *et al.*, 1971).

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