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Studies on the Subunit Structure of Ovine Brain Glutamine Synthetase*

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ABSTRACT: Ovine brain glutamine synthetase is isolated in an octameric form exhibiting a sedimentation coefficient, $s_{20,w}$, of 15.0 S and possessing a molecular weight of 525,000 ± 25,000. In the presence of 2 M urea at 25°, 1 M urea at 35°, 20% dimethylformamide, 20% dimethyl sulfoxide, or at values of pH greater than 8.1 in low ionic strength media, the enzyme dissociates reversibly to a form, probably a tetramer, exhibiting a sedimentation coefficient of 8.6 S. Such dissociation is prevented by adenosine triphosphate and Mg^{2+}. Association of the 8.6S species to re-form the octamer is promoted by removal of the dissociating agent or by addition of adenosine triphosphate and Mg^{2+}. Reversible dissociation in 1 M urea is temperature dependent. Studies at various values of pH suggest that a functional group of the enzyme with a pK of about 8.1 is involved in the reversible dissociation phenomenon. The data suggest that dissociation of the octamer to the 8.6S form does not involve extensive alteration of the tertiary structure of the enzyme, and that certain reagents are quite specific for disrupting the linkage between tetramers. The findings are consistent with a model of the octameric enzyme in which two heterologously linked tetramers are held together by weaker isologous bonds. Treatment of the octameric enzyme with maleic anhydride, acetic anhydride, tetranitromethane, diazonium-1H-tetrazole, or adjustment of the pH to values greater than 9.8 yields a catalytically inactive monomeric species [$s_{20,w}$ = 2.8 S; mol wt 65,000].

Glutamine synthetase has been isolated from sheep brain as an apparently homogeneous preparation exhibiting a molecular weight of about 525,000 (Pamiljans *et al.*, 1962; Ronzio *et al.*, 1969a; Haschemeyer, 1965). The enzyme is composed of 8 apparently identical subunits with a cubelike morphological appearance (Haschemeyer, 1965, 1966). Electron microscope studies and considerations of symmetry have led to the formulation of a model for the enzyme which possesses D_4 symmetry (Haschemeyer, 1968). Inhibition of glutamine synthetase by methionine sulfoximine is associated with the tight binding to the enzyme of 8 moles of methionine sulfoximine phosphate together with an equivalent quantity of ADP (Ronzio and Meister, 1968; Ronzio *et al.*, 1969b). In addition, the enzyme can bind with high affinity 8 moles of ATP and with less affinity a total of 16 moles of ATP (Wellner and Meister, 1966; Ronzio *et al.*, 1969b). The available data indicate that enzymatic activity resides in the native octameric enzyme. In the investigations reported here, the subunit structure of the enzyme has been examined by application of a number of procedures which produce dissociation of the octamer. Evidence has been obtained that the enzyme can be reversibly dissociated to yield a form exhibiting a sedimentation coefficient of 8.6 S and irreversibly to a monomeric component ($s_{20,w}$ = 2.8 S). The findings support the model proposed by Haschemeyer (1968).

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Experimental Section

Materials

Glutamine synthetase was isolated from sheep brain as described by Ronzio *et al.* (1969a); the preparations used here exhibited specific activities in the range 170–200 units/mg, and were stored in the dry lyophilized state at −20°. Under these conditions, some preparations of the enzyme developed small amounts of catalytically inactive very high molecular weight material; when present, such aggregates were removed from solutions of the enzyme by high-speed centrifugation.

Ultrapure urea was obtained from Mann Chemical Co. Adenosine triphosphate, adenosine diphosphate, and *N*-ethylmaleimide were obtained from Sigma Chemical Co. Tetranitromethane, 5-amino-1H-tetrazole, maleic anhydride, and

5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Aldrich Chemical Co.

Bio-Gel 300 P was obtained from Calbiochem, and Sephadex G-50 was obtained from Pharmacia Chemicals. The gels were suspended in 0.05 M potassium phosphate buffer (pH 7.2) for 24 hr prior to use. Visking dialysis tubing was prepared for use by treatment at 100° with water, 0.02 M EDTA, and 5% sodium bicarbonate, in sequence; it was then washed with copious amounts of distilled water until the wash water failed to exhibit absorbance at 280 mμ.

L-Methionine (*SR*)-sulfoximine and L-methionine (*SR*)-sulfoximine phosphate were obtained as previously described (Rowe *et al.*, 1969).

Methods

Glutamine synthetase activity was determined by measuring the amount of γ-glutamyl hydroxamate formed in the standard assay system previously described (Ronzio *et al.*, 1969a). Protein was determined by the procedure of Lowry *et al.* (1951) using bovine serum albumin as a standard, or from the absorbance of the enzyme at 280 mμ; a solution of the enzyme containing 1 mg/ml exhibits an absorbance of 1.35 (Ronzio *et al.*, 1969a).

Gel filtration was carried out with columns (0.2 × 16 cm) of Bio-Gel P-300 or Sephadex G-50; the enzyme solution (0.5 mg/0.5 ml) was added to the top of the column and elution was carried out with 0.01 M potassium phosphate buffer (pH 7.2) containing 0.01 M 2-mercaptoethanol and 0.002 M EDTA.

Electrophoresis on polyacrylamide gel was carried out by the procedure of Davis (1964); electrophoresis was carried out on 6 × 50 mm (6%) gels in a 0.05 M potassium phosphate buffer (pH 7.2) at 25° for 3 hr; 110 V; current, 50 mA. In some studies 2 M urea was added to both buffer and gel.

Determination of enzyme sulfhydryl groups was carried out by the procedure of Ellman (1959). In these studies the enzyme was dialyzed exhaustively at 4° against 0.01 M potassium phosphate buffer (pH 7.2) containing 0.002 M EDTA in order to remove 2-mercaptoethanol. Solutions of the enzyme (0.7 ml containing 0.35 mg) were added to a spectrophotometer cuvet and treated with 0.025 ml each of 0.25 M potassium phosphate buffer (pH 7.2) and 0.01 M EDTA. The absorbancies determined at 412 mμ, after addition of 0.02 ml of 0.01 M 5,5′-dithiobis(2-nitrobenzoate) in 0.05 M potassium phosphate buffer (pH 7.0), were corrected for the protein and reagent blanks. The corrected increase in absorbance at 412 mμ was determined as a function of time, and the corrected initial rate was extrapolated to zero time to obtain the number of instantaneously reacting sulfhydryl groups (Ronzio *et al.*, 1969a).

The effect of urea on the binding of methionine sulfoximine to the enzyme was studied using L-[^{14}C]methionine sulfoximine (Ronzio *et al.*, 1969b). An aliquot (0.6 ml) of enzyme in 0.01 M pH 7.2 Tris-HCl buffer containing 0.5 mg of protein was incubated for 15 min at 37° with 0.1 ml of L-[^{14}C]-methionine sulfoximine (1.27 μmoles; 1.50 × 10^6 cpm/μmole) in the presence of 0.02 M $MnCl_2$, 0.01 M ATP, and 0.01 M 2-mercaptoethanol. The enzyme–[^{14}C]methionine sulfoximine complex was separated from free [^{14}C]methionine sulfoximine by passage through Sephadex G-50. An aliquot (1 ml) of the complex was treated with 0.12 g of urea (final concentration 2 M) for 15 min at 25° and then passed through a second column of Sephadex G-50. Fractions were monitored for radioactivity by liquid scintillation counting (Ronzio *et al.*, 1969b).

Optical rotatory dispersion was performed with a Cary Model 60 spectropolarimeter. Ultraviolet difference spectra were taken with a Cary Model 14 spectrophotometer using the procedure described by Wetlaufer (1962).

The enzyme was treated with maleic anhydride as described by Sia and Horecker (1968). A similar procedure was followed in the studies with acetic anhydride. Reaction of the enzyme with diazonium-1H-tetrazole was carried out as described by Sokolovsky and Vallee (1966). Treatment of the enzyme with tetranitromethane (Sokolovsky *et al.*, 1966) was carried out by mixing a solution of the enzyme (0.5 mg/ml) with the reagent as described, followed by exhaustive dialysis against 0.01 M potassium phosphate buffer (pH 7.2) containing 0.01 M 2-mercaptoethanol and 0.002 M EDTA. Reaction of the enzyme with *N*-ethylmaleimide was carried out in the absence of 2-mercaptoethanol; the enzyme was incubated in 0.01 M potassium phosphate buffer (pH 7.2) containing 0.002 M EDTA and 0.005 M *N*-ethylmaleimide at 37° for 5 min.

Sedimentation coefficients were measured with a Spinco Model E ultracentrifuge equipped with an ultraviolet scanning system. A solution of the enzyme (usually 0.4 ml containing 0.2 mg of protein) was added to the right-hand compartment of a standard double sector cell containing an Epon centerpiece and equipped with quartz windows. The reference compartment was filled with the appropriate buffer. The cell was placed in an AN-D rotor, which was brought to 59,280 rpm; scans at 280 mμ were automatically recorded at 4-min intervals. In the experiments with ATP and ADP, it was not possible to use the ultraviolet optical scanning system of the ultracentrifuge at 280 mμ because of the high absorbance due to the nucleotides at this wavelength; therefore experiments in which nucleotides were present were carried out at 292 mμ. At this wavelength it is possible to observe the protein boundary in the presence of 0.01 M ATP. The sedimentation rate was computed from the least-squares slope of a plot of the logarithm of the displacement of the midpoint of the protein boundary from the center of rotation against time. When both 15S and 8.6S components were present, the relative amounts of these were estimated from the respective boundary heights on the recorded plots. The measurements carried out in solutions of urea were corrected to the viscosity and density of water at 20° using the equations given by Kawahara and Tanford (1966). Appropriate corrections for viscosity and density were applied in the studies carried out with dimethylformamide (Blankenship and Clampitt, 1950), acetonitrile (Vierk, 1950), dimethyl sulfoxide (LeBel and Goring, 1962), and tetrahydrofuran (Critchfield *et al.*, 1953; Blankenship, 1949; International Critical Tables, 1928). It was assumed that $\bar{V}_T = \bar{V}_{20} = 0.73$ cc/g.

The 2.8S form of the enzyme was prepared for molecular weight determination by reaction with maleic anhydride as described above. The dissociated enzyme was dialyzed against 0.1 M pH 7.2 potassium phosphate buffer containing 0.1 M NaCl. The preparation gave a single symmetrical boundary on analytical ultracentrifugation. The molecular weight was determined by a lower speed, higher concentration modification of the Yphantis (1964) method. The 8.6S form of the enzyme was prepared for molecular weight determination by treatment of the isolated enzyme with 2 M urea, followed by purification on a column of Bio-Gel P-300 equilibrated with

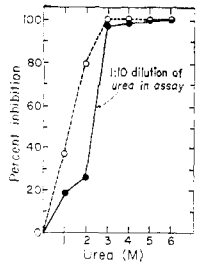

FIGURE 1: Effect of urea on glutamine synthetase activity. The enzyme (0.1 mg/ml) was incubated in solutions containing 0.01 M potassium phosphate buffer (pH 7.2), 0.002 M EDTA, 0.01 M 2-mercaptoethanol, and urea (in the indicated concentrations) at 25° for 3 hr. Upper curve (open circles): activity was determined in the presence of the same concentration of urea that was present during incubation at 25°, *i.e.*, as indicated in the figure. Lower curve (closed circles): activity was determined in the presence of one-tenth of the concentration of urea present during incubation.

2 M urea. The protein contained in the void volume peak was dialyzed against 2 M urea containing 0.1 M NaCl. Examination of the material by analytical ultracentrifugation revealed no undissociated (15.0 S) enzyme and approximately 6% trailing material. The molecular weight was determined by the meniscus depletion method of Yphantis (1964), at a speed of 13,416 rpm and a temperature of 5.8°.

Results

Effect of Urea on Glutamine Synthetase Activity. In an effort to obtain information about the relationship of subunit structure to activity, conditions were sought in which dissociation could be achieved without extensive disruption of the tertiary structure of the enzyme. Thus, the effect of various concentrations of urea on the activity of the enzyme was studied. In the experiments described in Figure 1, the enzyme was incubated with various concentrations of urea at 25° for 3 hr. After incubation, activity was determined at 37° in the presence of the same concentration of urea used in the incubation (upper curve) and also in the presence of one-tenth of this concentration (lower curve). In the first set of activity determinations, inhibition was essentially proportional to urea concentration up to 3 M urea. In the second series of experiments, in which a much lower concentration of urea was present during the determination of activity, much less inhibition was observed in the studies in which the enzyme was incubated with 1 and 2 M urea; on the other hand, incubation of the enzyme with concentrations of urea that were 3 M or higher led to irreversible inhibition. When the enzyme was incubated with 2 M urea for 3 hr and then extensively dialyzed, virtually all of the initial activity returned. Studies in which urea was added to the standard assay system indicated that urea did not inhibit activity at concentrations less than 0.9 M.

In the course of these studies it was found that the effect of urea on activity is much less marked on partially purified preparations of the enzyme (specific activity 50–70 units/mg). Furthermore, addition of bovine serum albumin (5 mg/ml) to a solution of purified glutamine synthetase (specific activity 185 units/mg) afforded complete protection against inactivation by 3 M urea after a 5-min exposure. However, such protection was found to be time dependent; thus, 70% inhibition was observed after the enzyme was incubated with serum albumin and 3 M urea for 24 hr. The inhibition of purified glutamine synthetase by urea is also time dependent; for example, the lower curve in Figure 1 was shifted to the left as the period of incubation of the enzyme with urea was increased. Neither EDTA nor 2-mercaptoethanol affected the inhibition of the purified enzyme by 2 M urea.

When samples of the purified enzyme (1 mg/ml) in 2 M urea were subjected to gel filtration as described under methods, two protein components were obtained, both of which exhibited glutamine synthetase activity. The first peak to emerge from the column appeared in the column volume and appeared to have a molecular weight equivalent to that of the native enzyme. The second component evidently possessed a lower molecular weight. There was some variability in the elution pattern obtained in studies of this type and a satisfactory estimate of the molecular weight of the second component could not be made. When polyacrylamide gel electrophoresis was carried out using the conditions described under methods, the native enzyme moved as a homogeneous band 2 mm toward the anode. When electrophoresis was carried out after treatment of the enzyme with 2 M urea, a more rapidly migrating band (13 mm) was found in addition to the major component exhibiting the mobility of the native enzyme. When 2 M urea was present in the buffer and in the gel, only a single rapidly migrating band (13 mm) was found after electrophoresis. These studies showed that the enzyme dissociates in the presence of 2 M urea to yield a form of the enzyme possessing a lower molecular weight. In order to pursue this phenomenon further, studies were carried out using the analytical ultracentrifuge.

Effect of Urea on the Sedimentation Coefficient of Glutamine Synthetase. The sedimentation coefficient of the native enzyme was found to be 15.0 S, a value in close agreement with earlier determinations (Pamiljans *et al.*, 1962; Haschemeyer, 1968). When the enzyme was treated with 2 M urea, there was complete dissociation of the native enzyme to yield a new species exhibiting a sedimentation coefficient of 8.6 S (Table I). After dialysis of the 2 M urea-treated enzyme there was complete reassociation to the 15S form of the enzyme (Table I, expt 3). Dissociation by 2 M urea was unaffected by the presence of either 0.002 M EDTA or 0.01 M 2-mercaptoethanol. On the other hand, only about 70% dissociation was observed in the presence of 2 M urea and 0.1 M sodium chloride. When the enzyme was treated with 1 M urea at 20° both 8.6S and 15.0S components were found with the latter predominating (Table I, expt 7). However, when the temperature was increased to 35°, the relative amount of 8.6S component increased; this phenomenon is reversible (Table I, expt 8). Thus, when the temperature was brought back to 20° much more of the 15S component was found, and after further decrease in temperature to 6°, only the 15S species was present. The findings indicate, therefore, that in 1 M urea there is a temperature-dependent equilibrium between the 15S and 8.6S forms of the enzyme. Similarly, at 25° there is a urea concentration-dependent equilibrium.

The weight-average molecular weight value for the 8.6S form of the enzyme, determined by the method of Yphantis (1964), was 185,000. Although this is less than 263,000 (theory for the tetramer), the preparation was devoid of the 15S form but contained a significant amount of the monomer. The

TABLE I: Effect of Urea and Temperature on the Sedimentation Coefficient, $s_{20,w}$, of Glutamine Synthetase.

Experiment[a]	Sedimentation Coefficient % 15 S	% 8.6 S
1. Control	100	0
2. 2 M urea	0	100
3. 2 M urea; then dialyzed[b]	100	0
4. 2 M urea (EDTA omitted)	0	100
5. 2 M urea (2-mercaptoethanol omitted)	0	100
6. 2 M urea; 0.1 M NaCl	30	70
7. 1 M urea; 20°	80	20
8.[c] (a) 1 M urea; 35°	20	80
(b) 20°	70	30
(c) 6°	100	0

[a] These studies were carried out with solutions of the enzyme (0.2 mg/0.4 ml) in 0.01 M potassium phosphate buffer (pH 7.2) containing 0.01 M 2-mercaptoethanol and 0.002 M EDTA. Sedimentation coefficients were determined as described under Methods. The relative amounts of the 15S and 8.6S components were calculated from the respective boundary heights on the recorded plots. Experiments 1–6 were carried out at 25°. [b] Dialyzed for 24 hr at 5° against 0.05 M potassium phosphate buffer containing 0.01 M 2-mercaptoethanol and 0.002 M EDTA. [c] In expt 8, sedimentation was carried out at 35° (a); the same solution was used (after mixing) for the studies at 20° (b) and 6° (c).

TABLE II: Effect of Substrates on the Sedimentation Coefficient of Glutamine Synthetase.

Experiment[a]	Sedimentation Coefficient % 15 S	% 8.6 S
1. ATP, Mg^{2+}, glutamate; 35°[b]	100	0
2. ATP, Mg^{2+}, glutamate;[c] then 1 M urea added; 35°	80	20
3. 1 M urea; then ATP, Mg^{2+}, glutamate[c] added; 35°	80	20
4. 1 M urea; 35°	15	85
5. ADP, Mn^{2+}; then 1 M urea added; 35°	50	50
6. ATP, Mg^{2+}, methionine sulfoximine; 25°	40[d]	0
7. ATP, Mg^{2+}, methionine sulfoximine; then 2 M urea added; 25°	33[e]	0
8. Methionine sulfoximine phosphate; then 2 M urea; 25°	15	85
9. 2 M urea; 25°	0	100
10. Mg^{2+}; then 2 M urea; 25°	0	100

[a] These studies were carried out on solutions of the enzyme (0.2 mg/0.4 ml) in 0.01 M potassium phosphate buffer (pH 7.2) containing 0.01 M 2-mercaptoethanol and 0.002 M EDTA; NaATP (0.01 M), NaADP (0.01 M), $MgCl_2$ (0.02 M), $MnCl_2$ (0.02 M), sodium L-glutamate (0.05 M), L-methionine (*SR*)-sulfoximine (0.01 M), and L-methionine (*SR*)-sulfoximine phosphate (0.01 M) were added as stated in the table. The solutions were incubated for 15 min at 37° prior to second addition (if any) and determination of sedimentation coefficient. [b] In the studies in which the temperature was set at 35°, there was about a 10% variation between replicate runs apparently related to fluctuations of temperature (±2°). [c] Similar results were obtained in the presence and absence of glutamate. [d] The sedimentation coefficient of the major component (about 40% of the total) was 15 S; one component (about 20%) exhibited a sedimentation coefficient of 22 S, and the remainder sedimented more rapidly. [e] Major component (33%), 15 S; (15%), 22 S; the remainder of the material sedimented more rapidly.

amount of trailing material increased from 6% at the start of the run to 23% at the end. Analysis of the data obtained clearly reflected the presence of the monomer (2.8 S).

Effect of Substrates on the Sedimentation Coefficient of Glutamine Synthetase. The gel filtration experiments described above suggest that the lower molecular weight component exhibits glutamine synthetase activity. However, since the determination of enzymatic activity is necessarily carried out in the presence of glutamate, ATP, and magnesium ions, it is necessary to know whether these components of the assay system can affect the state of aggregation of the enzyme. Sedimentation studies showed that the enzyme, after brief incubation with ATP, Mg^{2+}, and glutamate, is completely in the 15S form at 35° (Table II). When 1 M urea was added to the substrate-treated enzyme (Table II, expt 2), only about 20% dissociation occurred. Similar results were obtained when 1 M urea was added prior to the substrates. In contrast, in the absence of substrates, 1 M urea produced 80–85% dissociation under these conditions (Table I, expt 8a; Table II, expt 4). Such prevention of dissociation was also observed in the presence of ATP and Mg^{2+} alone; *i.e.*, similar results were obtained in the presence and absence of glutamate. Furthermore, neither ATP nor Mg^{2+} alone prevented dissociation. About 50% dissociation was observed when ADP and Mn^{2+} and 1 M urea were present. When the enzyme was incubated with ATP, Mg^{2+}, and methionine sulfoximine there was no dissociation, and only 40% of the protein was present in the 15S form; the remainder was present partly as an apparent dimer of the native enzyme ($s_{20,w}$ = 22 S) and partly as higher molecular weight material. In the presence of ATP, Mg^{2+}, methionine sulfoximine, and 2 M urea there was no dissociation and indeed a somewhat greater tendency to aggregation was observed. When enzyme containing bound [^{14}C]methionine sulfoximine was treated with 2 M urea and then passed through a column of Sephadex G-50, all of the radioactivity was found associated with the enzyme. Electron microscope studies of the enzyme after treatment with methionine sulfoximine, ATP, and Mg^{2+} show extensive aggregation of the octameric units to form long chains (R. H. Haschemeyer, unpublished data 1969). When the enzyme was treated with methionine sulfoximine phos-

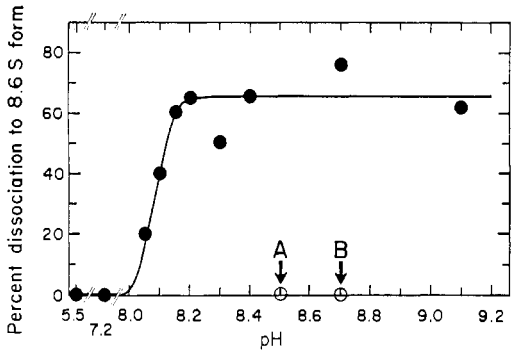

FIGURE 2: Effect of pH on the dissociation of glutamine synthetase. The enzyme (0.5 mg/ml) in 0.05 M potassium phosphate buffer containing 0.01 M 2-mercaptoethanol and 0.002 M EDTA was dialyzed at 25° for 12 hr against buffers of various values of pH; then 0.4 ml of the enzyme solution was subjected to analytical ultracentrifugation. The buffers used were: sodium acetate (0.05 M; pH 5.50), potassium phosphate (0.01 M; pH 7.20), Tris-HCl (0.02 M; pH 8.05, 8.10, 8.15, 8.20, 8.30, and 8.40), potassium phosphate (0.02 M; pH 8.70), sodium borate–carbonate (0.05 M; pH 8.15), glycine–NaOH (0.05 M; pH 9.10). (A) pH 8.50; sodium borate–carbonate (0.05 M) containing 0.1 M NaCl. (B) pH 8.70; potassium phosphate (0.02 M); incubated with 0.01 M ATP and 0.02 M $MgCl_2$ for 15 min at 37°.

phate and 2 M urea at 25° (Table II, expt 8), there was substantial dissociation to the 8.6S form; however, dissociation was significantly less than observed with 2 M urea alone. The findings indicate that methionine sulfoximine phosphate inhibits to some extent the urea-induced dissociation phenomenon and that the extensive aggregation observed when the enzyme is treated with methionine sulfoximine, ATP, and Mg^{2+} is associated with the presence of nucleotide and metal ions. Other studies (Rowe *et al.*, 1969) have shown that the enzyme binds methionine sulfoximine phosphate much less tightly in the absence of ADP and Mg^{2+} than in their presence.

Dissociation of the Enzyme by Dimethylformamide and Dimethyl Sulfoxide. The effect of dimethylformamide and dimethyl sulfoxide on the dissociation of glutamine synthetase is described in Table III. In the presence of 25% dimethylformamide at 25° the enzyme was about 90% dissociated to the 8.6S form. Removal of the dimethylformamide by dialysis resulted in restoration of the 15S form. No dissociation was observed under these conditions in the presence of 5 or 15% dimethylformamide. When the concentration of dimethylformamide was increased to 50% or when 25% dimethylformamide was used at 35° there was considerable irreversible aggregation and denaturation of the enzyme. It is of interest that the presence of ATP, Mg^{2+}, and methionine sulfoximine inhibited dissociation by dimethylformamide. Similar results were obtained with dimethyl sulfoxide; thus, substantial dissociation of the native enzyme to the 8.6S form of the enzyme was observed with 20% dimethyl sulfoxide, and this was prevented by ATP and Mg^{2+}.

Treatment of the enzyme with 20% 2-chloroethanol, 20% tetrahydrofuran, or 20% acetonitrile failed to induce dissociation to a stable intermediate; on the contrary, these reagents led to rapid extensive aggregation and denaturation (with precipitation) of the enzyme.

Reversible Dissociation of the Enzyme at Alkaline Values of pH. When the pH of the enzyme solution was brought to values greater than 8.1, reversible dissociation to the 8.6S form took place. Such dissociation was found to be highly dependent upon ionic strength; thus dissociation at pH 8.5 was prevented by addition of 0.1 M sodium chloride. The relationship between per cent dissociation of the enzyme and pH (Figure 2) bears a resemblance to a titration curve exhibiting a midpoint at about pH 8.1. Dissociation at alkaline pH was reversed when the pH was brought back to values less than 8.1 and also when ATP and Mg^{2+} were added.

TABLE III: Effect of Dimethylformamide (DMF) and Dimethyl Sulfoxide (DMSO) on the Sedimentation Coefficient of Glutamine Synthetase.[a]

Experiment	Sedimentation Coefficient: % 15 S	Sedimentation Coefficient: % 8.6 S
1. 5% DMF	100	0
2. 15% DMF	100	0
3. 25% DMF	10	90
4. 25% DMF; then dialyzed[b]	100	0
5. 50% DMF	0[c]	0
6. 25% DMF; 35°	0[c]	0
7. ATP, Mg^{2+}, methionine sulfoximine; then 25% DMF[d]	66[f]	0
8. 20% DMSO	25	75
9. ATP, Mg^{2+}, then DMSO, 20%[e]	100	0

[a] These studies were carried out on solutions of the enzyme (0.2 mg/0.4 ml) in 0.01 M potassium phosphate buffer (pH 7.2) containing 0.01 M 2-mercaptoethanol, 0.002 M EDTA, and dimethylformamide or dimethyl sulfoxide in the concentrations (v/v) indicated. The temperature was 25° unless otherwise stated. [b] Dialyzed against 0.05 M potassium phosphate buffer (pH 7.2) containing 0.01 M 2-mercaptoethanol and 0.002 M EDTA. [c] Marked aggregation to form several species with $s_{20,w}$ greater than 15 S. [d] Incubated with 0.01 M ATP, 0.02 M $MgCl_2$, and 0.01 M L-methionine (*SR*)-sulfoximine at 37° for 15 min; then 25% (final concentration) DMF was added. [e] Incubated with 0.01 M ATP, and 0.02 M $MgCl_2$ at 37° for 15 min; then 20% (final concentration) DMSO was added. [f] See Table II, expt 6.

Irreversible Dissociation of the Enzyme to Monomers. Treatment of the enzyme with any one of a variety of reagents has been found to produce complete dissociation to a single homogeneous component that exhibited a sedimentation coefficient of 2.8 S. Such dissociation was not reversed by dialysis and the monomeric unit obtained in this manner was completely devoid of enzymatic activity. The molecular weight of the 2.8S form of the enzyme produced by treatment with maleic anhydride was found to be 65,000, thus identifying this component as the monomer. Dissociation of the 15S form of the enzyme to the 2.8S form was also accomplished by treatment with an excess of (a) diazonium-1H-tetrazole, (b) tetranitromethane, (c) acetic anhydride, and (d) adjustment of the pH to values greater than 9.8.

Studies on the Structure and Properties of the 8.6S Form of

the Enzyme. Previous studies have shown that native glutamine synthetase contains close to 8 moles of highly reactive sulfhydryl groups as determined by titration with 5,5′-dithiobis(2-nitrobenzoate). When this determination was carried out at 25° as previously described (Ronzio *et al.*, 1969a), in the presence of 1 M urea, *i.e.*, under conditions in which appreciable dissociation does not occur, a similar value for rapidly reacting sulfhydryl groups was obtained. When the titration was carried out at 25° in 2 M urea, *i.e.*, a condition which leads to complete dissociation of the 15S form to the 8.6S form, no additional exposure of instantaneously reacting sulfhydryl groups occurred, although the rate of reaction of additional groups was observed to increase. Similar studies in 6 M urea led to instantaneous exposure of about 77 moles of sulfhydryl groups/mole of enzyme.

Optical rotatory dispersion studies (220–300 mμ) were carried out on the 15S and 8.6S forms of the enzyme and the corresponding b_0 values were calculated (Urnes and Doty, 1961); nearly identical values (b_0 = 190 and 191, respectively) and curves were obtained for both forms of the enzyme. When optical rotatory dispersion was carried out in 6 M urea, the b_0 value decreased to zero.

Ultraviolet difference absorption spectra (2 M urea-treated enzyme *vs.* the isolated enzyme) showed no significant differences. On the other hand, characteristic evidence of marked exposure of chromophoric groups was evident when the enzyme was examined in this manner after treatment with 6 M urea. The findings indicate that the reversible dissociation of the enzyme does not involve major changes in its tertiary structure.

Reaction with N-Ethylmaleimide. Incubation of the enzyme with 5 mM *N*-ethylmaleimide for 5 min at 37° led to complete inhibition which was not reversed by dialysis. Such inhibition was prevented by preincubation with 0.01 M ATP and 0.02 M Mg^{2+}; 50% protection was afforded by 0.0011 M ATP, a value close to the observed K_m value for ATP. When larger amounts of enzyme, required for ultracentrifugal analysis, were incubated with 5 mM *N*-ethylmaleimide, precipitation of the enzyme was observed. Such precipitation was not evident when the enzyme was preincubated with ATP and Mg^{2+}. Examination of mixtures of enzyme and *N*-ethylmaleimide by analytical ultracentrifugation revealed extensive aggregation to discrete higher molecular weight forms. No dissociation was observed.

Discussion

The present studies are in agreement with the earlier findings (Pamiljans *et al.*, 1962; Haschemeyer, 1965, 1966, 1968) that ovine brain glutamine synthetase as isolated exhibits a sedimentation coefficient $s_{20,w}$ of 15.0 S; this form of the enzyme has a molecular weight of about 525,000. Previous studies (Haschemeyer, 1965, 1966, 1968) have shown that the isolated enzyme has 8 subunits with a cubelike morphological appearance, and the available data are in accord with the view that the subunits are identical (Ronzio *et al.*, 1969a). The findings reported here indicate that the octameric enzyme dissociates to a form exhibiting a sedimentation coefficient of 8.6 S in the presence of 2 M urea at 25°; such dissociation is prevented by ATP and Mg^{2+} and may be reversed by removal of urea. Dissociation also occurs in 1 M urea at 35° and this can be reversed by decreasing the temperature, whereas in 2 M urea at 35° irreversible dissociation takes place. The data show that ATP in the presence of Mg^{2+} prevents dissociation by urea, tetrahydrofuran, dimethyl sulfoxide, and increase of pH. Earlier studies have indicated that the activity of the enzyme is rapidly lost at values of pH greater than about 8. It is of interest that ATP and Mg^{2+} protect the enzyme against heat denaturation (Pamiljans *et al.*, 1962) and denaturation by *N*-ethylmaleimide (Ronzio *et al.*, 1969a). The concentration of ATP that affords 50% protection against such inactivation is not far from the K_m value for ATP for glutamine synthetase activity. Previous work (Ronzio *et al.*, 1969a) on the sulfhydryl groups of the enzyme demonstrated the presence of one highly reactive SH residue per subunit and 12–14 more slowly reacting SH groups which are presumably buried within the protein. Reaction of the enzyme with [^{14}C]*N*-ethylmaleimide in the presence of ATP and Mg^{2+} led to incorporation of radioactivity equivalent to about one alkylated residue per subunit. Reaction in the absence of ATP and Mg^{2+} led to extensive aggregation and subsequent precipitation of the enzyme, suggesting progressive reaction and unfolding of the peptide chain exposing additional reactive groups. The fact that this process does not occur in the presence of nucleotide and metal ion emphasizes their role in the stabilization of the native conformation of the octamer.

It is notable that the conversion of the 15S to the 8.6S form is specifically promoted by a number of reagents (urea, dimethylformamide, and dimethyl sulfoxide), while other denaturants (tetrahydrofuran and acetonitrile) are apparently less specific. The results obtained with these reagents have been useful in the structural studies *per se* and may also be of significance in elucidation of the nature of the intersubunit bonds. The studies carried out at various values of pH (Figure 2) suggest that a functional group possessing a pK of about 8.1 may be involved in the reversible association–dissociation phenomenon. Dissociation evidently involves no major disruption of the tertiary structure of the enzyme. Thus, no significant changes were noted on dissociation in the number of instantaneously reacting sulfhydryl groups, optical rotatory dispersion, or ultraviolet absorption difference spectrum. Using the relationship $[M_1]^{2/3}/[M_2]^{2/3} = S_1/S_2$, derived from the equations of Scheraga and Mandelkern (1953), one may calculate "theoretical" sedimentation coefficients of 9.0, 5.8, and 3.7 S for the tetramer, dimer, and monomer, respectively (assuming axial ratios of 2.0, 2.0, and 1.0, respectively). The value of 8.6 S obtained in the presence of perturbing solvents is slightly lower than the "theoretical" value for a tetramer suggesting that the "reversible" tetramer may be somewhat more hydrated or slightly extended. The inactive monomer obtained by relatively drastic methods exhibits a sedimentation coefficient of 2.8 S suggesting that this form may exist in a less compact conformation.

The value for the weight-average molecular weight of the 8.6S form (185,000), is greater than theory for a dimer (131,000) but is less than expected for a tetramer (263,000). However, the absence of the 15S species and the finding of at least 20% monomer in the sample used for analysis would be expected to decrease the observed weight-average molecular weight value. The weight of evidence available therefore supports the tentative conclusion that the "reversible 8.6S form" is a tetramer. The present findings are in accord with the view that enzyme activity resides in the octameric enzyme. Because ATP and Mg^{2+} strongly promote association of the "8.6S tetramer,"

it has not thus far been possible to determine whether this form of the enzyme itself exhibits catalytic activity.

The data presented here are consistent with the model previously proposed (Haschemeyer, 1968) for the native enzyme, *i.e.*, an octamer possessing D_4 symmetry (Monod *et al.*, 1965; Hanson, 1966). According to this model, the octamer is formed by the isologous association of two heterologously bonded tetramers. We thus tentatively conclude that the heterologous tetramers of the native octamer are linked together by isologous bonds which are less stable to perturbation by the dissociating solvents used in these studies. The data indicate clearly that nucleotide and metal ion confer considerable stability on the octamer. It remains to be determined whether such stability is conferred by direct interaction near subunit bonding sets or is the result of a more distant conformation-induced change in bonding set geometry.

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