The Regulation of Glutamine Synthesis in the Food Yeast *Candida utilis*: The Purification and Subunit Structure of Glutamine Synthetase and Aspects of Enzyme Deactivation

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SUMMARY

Yeast glutamine synthetase was purified and shown to be an octameric globular protein ($s = 15.4$, $f/f_0 = 1.29$, mol. wt = 390000). It consists of two weakly-bound half molecules ($s = 8.7$, $f/f_0 = 1.35$, mol. wt = 180500) and relatively harsh treatment is required to dissociate these octamers into component monomers ($s = 3.8$). Deactivation of the enzyme *in vivo* may include changes in the conformation of the native enzyme with its separation into two 'tight' tetramers, followed by their dissociation into component monomers and dimers. In the presence of Mg^{2+} and glutamate, monomers re-aggregate to oligomers which, although having transferase activity, are devoid of synthetase activity. The relevance of these observations in relation to control of glutamine synthetase activity in yeast is discussed.

INTRODUCTION

Previous studies on the glutamine synthetase of *Candida utilis* have revealed that the enzyme is subject to rapid and extensive deactivation (Ferguson & Sims, 1971). Modulation of enzyme concentration, achieved mainly by irreversible deactivation and rapid resynthesis, is primarily responsible for the regulation of glutamine synthesis (Sims & Ferguson, 1972; 1974; Ferguson & Sims, 1974*a, b*). These observations leave unanswered the question of how such precise adjustment of enzyme concentration is achieved. In the present paper we examine the subunit structure of the yeast glutamine synthetase and its possible influence on a mechanism for enzyme deactivation.

METHODS

Organism and culture conditions. *Candida utilis* NCYC 737 was grown as described earlier (Ferguson & Sims, 1971).

Assay for enzyme activity. Glutamine synthetase [L-glutamate:ammonia ligase (ADP) EC. 6.3.1.2] was measured as transferase activity and synthetase activity (see Ferguson & Sims, 1971; 1974*a*).

Measurement of radioactivity. The scintillation fluid contained 2,5-diphenyloxazole (3 g) and 1,4-bis-[2(4-methyl-5-phenyl-oxazol-2-yl)]benzene (0.3 g) in toluene (1 l) and Triton X-100 (500 ml). Samples, made to 1 ml with water, were added to 12 ml of scintillation fluid and counted in a Packard Tricarb scintillation spectrometer. The d.p.m. were computed from the c.p.m. by a channel ratio method as described by Glass (1970). The efficiency of counting was about 70 %.

Method for determining the sedimentation behaviour of glutamine synthetase. The sucrose method of Martin & Ames (1961) was used. Four well-characterized crystalline enzymes of different sedimentation coefficients were chosen as standards; β -galactosidase from *Escherichia coli* $s_{20,w} = 16.1$; bovine liver catalase $s_{20,w} = 11.3$; yeast alcohol dehydrogenase $s_{20,w} = 7.4$ and equine myoglobin $s_{20,w} = 2.0$.

Gel filtration. Gel filtration of glutamine synthetase and products of its dissociation was by conventional chromatographic procedures on Sepharose 6B (Pharmacia, Uppsala, Sweden). The swollen gel was crudely fractioned by sedimentation and the 'fines' packed into a column at the operating temperature (4°C). The glass column, 30×1 cm, was fitted at both ends with fine nylon mesh. The buffer in all experiments was 0.05 M-potassium phosphate (pH 7.5) + 0.1 M-NaCl. A 0.3 ml sample of enzyme was applied and eluent fractions (about 0.45 ml) were collected into tubes and weighed to obtain accurate values of elution volumes. The column was calibrated using crystalline enzymes with well-characterized Stokes's radii, a (see Ackers, 1964; Siegel & Monty, 1966): thyroglobulin, $a = 8.1$ nm; β -galactosidase from *Escherichia coli*, $a = 6.85$ nm; bovine L-glutamate dehydrogenase, $a = 6.4$ nm; jackbean urease monomer, $a = 6.19$ nm; bovine liver catalase, $a = 5.22$ nm; yeast alcohol dehydrogenase, $a = 4.55$ nm; rat liver lactic dehydrogenase, $a = 3.74$ nm; bovine myoglobin, $a = 1.88$ nm. The gel filtration data were plotted according to the correlation of Laurent & Killander (1964). Marker enzymes were added to glutamine synthetase extracts prior to analysis. Determinations were carried out in triplicate and values for the elution volume of the enzyme did not differ by more than 0.1 ml.

Calculation of molecular weights. The molecular weight and the frictional ratio of glutamine synthetase can be calculated provided the Stokes's radius, sedimentation coefficient and the partial specific volume of the enzyme are known. The techniques of gel filtration and sucrose gradient centrifugation provide a fairly reliable means by which estimates of Stokes's radius and sedimentation coefficients can be obtained with crude enzyme preparations. The error in the determination of the molecular weight in these experiments is estimated to be about 8%. The partial specific volume (v) of the glutamine synthetase has not been determined; pig brain glutamine synthetase has been shown (Stahl & Jaenicke, 1972) to have $v = 0.721$ $\text{cm}^3 \text{g}^{-1}$ and this value has been used.

A very approximate estimation of the molecular weight of a globular protein can also be obtained directly from the sedimentation coefficient (Schachman, 1959) and the molecular weights of dissociation products of glutamine synthetase have been determined in this way. However, rather than relying upon the relationship between sedimentation coefficient and molecular weight of a single standard protein, the sedimentation coefficients for a large number of proteins (data obtained from Klotz & Darnell, 1969) were plotted against their corresponding (mol. wt) $^{2/3}$. The line of best fit was calculated for the data and the molecular weight for the glutamine synthetase subunits determined from this graph.

Purification of glutamine synthetase. A small-scale method for the purification of yeast glutamine synthetase was developed that is particularly suitable for making isotopically labelled enzyme (see Table 1). Yeast grown on medium containing glucose as carbon source and glutamate as the sole source of nitrogen (up to 3 l of culture) was harvested by suction filtration on Whatman GF/A paper. The filter pad with yeast (1 to 3 g dry wt) was frozen in liquid nitrogen and the yeast was broken in a stainless steel hammer press chilled in liquid nitrogen. The debris was mixed with 10 ml phosphate buffer (5 mM, pH 7.2) containing 0.5 mM-EDTA and 50 mM- K_2SO_4 , and unbroken yeast and particulate matter were removed by centrifuging at 20000 g for 15 min.

For the preparation of enzyme labelled with $[\text{U}-^{14}\text{C}]$ isoleucine, yeast growing exponen-

Table 1. Purification of glutamine synthetase from glutamate grown cells

Protein fraction	Specific activity* ($\mu\text{mol/min/mg}$ protein at 30 °C)	Percentage recovery†
Crude untreated extract	1.22	100
Ammonium sulphate precipitate	2.01	95
DE-52 eluant	12.4	60
Sucrose gradient	75.0	48

* Estimated by transferase assay.

† Typical results given but the recoveries obtained in final two steps varied somewhat depending on how the fractions were bulked.

tially on glucose and glutamate was transferred to fresh culture medium (normally 1 l), containing glutamate (2.8 mM) and [^{14}C]isoleucine (30 μCi , 0.15 mM) plus other nutrients, and allowed to grow for a further 2 h. Yeast extracts were then prepared as above. Extracts were then subjected to $(\text{NH}_4)_2\text{SO}_4$, with material precipitating between 35 and 55 % (w/v) saturation being taken up into the minimum quantity of 0.05 M-tris buffer (pH 7.3). The protein fraction was then passed through a column (20 $\times$ 1 cm) of Sephadex G-25 equilibrated with 0.05 M-tris buffer (pH 7.3) and the appropriate fractions loaded on a column (4 $\times$ 1.2 cm) of Whatman DE 52. The column was eluted step-wise with 10 ml of 0.05 M-tris buffer (pH 7.3), 16 ml of 0.05 M-tris buffer (pH 7.3) + 0.06 M-NaCl, and finally with 0.05 M-tris buffer (pH 7.3) + 0.09 M-NaCl. Most enzyme activity was eluted with between 4 and 6 ml of the final buffer.

Up to 350 μl of the bulked fractions of the eluant from the DE 52 column were layered on a linear (5 to 20 %) sucrose gradient and purified by centrifuging for 15 h at 105 000 g. The fractions containing the greater part of the enzyme (Fig. 1a) were bulked and freed of sucrose by passage through a pre-cooled column (6 $\times$ 0.8 cm) of Sephadex G-25 (ultra-fine) equilibrated with ice-cold 0.05 M-tris buffer (pH 7.3). The overall enrichment of enzyme from extracts of glutamate de-repressed yeast is about 75-fold, with a recovery of nearly 50 % of the original activity (see Table 1).

A very large increase in the specific activity of the enzyme occurred at the final step of the purification procedure. Since many of the protein impurities in the DE 52 column eluant had sedimentation coefficients below 15 s (see Fig. 1), a simple large-scale method for purification of this enzyme could be to replace the final sucrose gradient step with molecular exclusion chromatography (Biogel 300 or 10 % agarose appear suitable).

When purified ^{14}C -labelled glutamine synthetase was fractionated on a sucrose gradient (Fig. 1b) the enzyme moved as a single symmetrical peak of nearly constant specific activity. The specific activity of the yeast enzyme was similar to that of crystalline glutamine synthetase from *Escherichia coli* (Woelfolk, Shapiro & Stadtman, 1966), provided due allowance was made for differences in the temperature of assay (see Table 1).

RESULTS

Determination of the molecular weight and frictional ratios of yeast glutamine synthetase

Sedimentation on sucrose gradients. When glutamine synthetase was sedimented on a sucrose gradient, all the catalytic activity was confined to a single symmetrical peak with a sedimentation coefficient of about 15.4 s. The enzyme was detected as both synthetase and

Fig. 1. Sucrose density gradient centrifugation of yeast glutamine synthetase labelled with $[\text{U-}^{14}\text{C}]$ -isoleucine under different conditions. (a) Partially purified enzyme centrifuged on a 5 to 20% (w/v) sucrose gradient. Fractions were assayed for transferase activity (Δ), ^{14}C content ($\blacksquare$) and protein ($\circ$). (b) Sedimentation behaviour of purified glutamine synthetase. The enzyme was fractionated on a sucrose gradient with four 'marker' enzymes ($\circ$) (myoglobin, yeast alcohol dehydrogenase, catalase and β -galactosidase) and assayed for transferase activity (Δ) and ^{14}C content ($\blacksquare$). (c) Sedimentation behaviour of purified glutamine synthetase on a sucrose gradient + 1 M-urea. Standard proteins were again included ($\circ$). Fractions were assayed for transferase activity (Δ) and ^{14}C content ($\blacksquare$).

transferase activity and gave a synthetase to transferase activity ratio of about 0.2 (Fig. 1*b*). In other experiments, crude enzyme extracts prepared from yeast grown on glutamate and a number of other amino acids as sole source of nitrogen gave identical values of the sedimentation coefficient and synthetase to transferase activity ratio. There were some indications of slight traces of activity in fractions corresponding to a sedimentation coefficient of about 9 with most of these crude enzyme preparations (see Table 2).

Gel filtration. The elution profile resulting from the chromatography of purified enzyme is shown in Fig. 2. The sepharose column used had previously been calibrated and a mean value of the Stokes's radius of the enzyme was estimated to be 6.23 nm.

Calculation of molecular parameters. Using the experimental values $s = 15.4$ and $a = 6.23$ nm, and $0.721 \text{ cm}^3 \text{ g}^{-1}$ as a value of v , the molecular weight of the yeast glutamine synthetase was calculated as 394000 ± 30000 and its molar friction coefficient as 1.29 (Table 2). This is therefore similar to the molecular weight of the enzyme from pig brain (Stahl & Jaenicke, 1972), rat liver (Tate, Leu & Meister, 1972) and *Neurospora crassa* (Kapoor, Bray & Ward, 1969). The molecular weight assigned to the enzyme from *Escherichia coli* is about 592000 (Shapiro & Ginsburg, 1968), indicating that the bacterial enzyme is likely to be structurally different.

Determination of the subunit structure of yeast glutamine synthetase

Other workers have shown that the bacterial and fungal glutamine synthetase could be readily dissociated on sucrose gradients containing 1 M-urea (Woolfolk & Stadtman, 1967; Kapoor *et al.* 1969). We have layered purified yeast enzyme on a 5 to 20 % sucrose gradient containing 1 M-urea in 0.05 M-tris buffer (pH 8.7) and, although a small peak of activity was observed in a position corresponding to a sedimentation coefficient of 14.2 *s*, the majority of the enzyme activity was located at about 8.7 *s* (Fig. 1*c*). The synthetase to transferase ratio of both species resembled the parent protein. The fractions associated with the 8.7 *s* component were mixed, desalted and chromatographed on Sepharose 6B. The Stokes's radius of the molecular species was established as 5.04 nm, which means that it has a molecular weight approximately one-half that of the parent enzyme (the calculated figure was 180400 ± 14000 , see Table 2). In some experiments nearly 50 % of the transferase activity applied to the urea gradient was recovered as 8.7 *s* component. This can be shown to correspond to an almost quantitative recovery of the enzyme as its tetramer and means that the enzyme preferentially dissociates into two halves under these conditions.

Enzyme labelled with [U- ^{14}C]isoleucine was used to establish whether the parent enzyme contained subunits of lower molecular weight. The pattern of labelling observed on a sucrose-urea gradient (Fig. 1*c*) showed that at least two additional components with smaller sedimentation coefficients were formed; unfortunately the resolution obtained did not permit any estimation of their values to be made. Prolonged incubation of these fractions with enzyme substrates produced an activity profile which corresponded quite closely to the pattern of ^{14}C label and indicated that these products could be detected by direct enzymic assay. We do not know whether this activity arises from the subsequent reassociation of catalytically inactive components during assay.

Any measurements of the sedimentation coefficients of the products of enzyme dissociation obtained by the direct application of native enzyme to sucrose gradients containing urea must be inaccurate, since the value obtained will depend upon the rate of dissociation of the enzyme in the gradient. Conditions were therefore established to dissociate the enzyme prior to analysis on the ultracentrifuge (Fig. 3). Short exposure of purified (15.4 *s*) enzyme to urea

Fig. 2. Gel filtration of yeast glutamine synthetase. (a) Analysis of a crude deactivated enzyme extract prepared by transferring yeast growing on glutamate to a medium containing NH_4^+ for 1 h. Fractions were analysed for transferase activity at pH 6.5 (Δ) and pH 5.8 ($\bullet$). (b) Analysis of purified enzyme ($\blacktriangledown$) together with the elution profile of the 14.2 s component isolated by sucrose density centrifugation of deactivated enzyme ($\square$). (c) Analysis of the enzyme tetramers. The 8.7 s component was prepared by layering purified enzyme on a sucrose gradient + 1 M-urea ($\blacksquare$) and the same prepared from crude deactivated enzyme extracts ($\bullet$). (d) Calibration of the Sepharose 6B column. The K_{av} values were determined from the elution positions for various crystalline proteins of known Stokes's radii, and the data treated according to Laurent & Killander (1964).

(1 M or 2 M) or 0.05 M-tris (pH 9.6) at 30 °C did not result in any appreciable loss of catalytic activity. However, treatment of the enzyme with a combination of 0.05 M-tris and 1 M-urea, under otherwise identical conditions, resulted in virtually a complete loss of catalytic activity. The inclusion of 60 mM-glutamate + 5 mM-ATP in the buffer afforded almost complete protection against enzyme inactivation. Analysis of the enzyme, removed after different times of exposure to urea at pH 9.6, established that denaturation was due to the dissociation of the enzyme: as denaturation proceeded, a decrease in the 14.2 s species was observed coincident with an increase in components with sedimentation coefficients of 5.8 and 3.8 s (see Table 2). Similar results were obtained when 0.03 M-sodium dodecyl sulphate was used to dissociate the enzyme. These sedimentation coefficients were used to calculate the approximate molecular weight for the smaller subunits. The values obtained (45500 and 90700) correspond to almost exact fractions of the weight of the parent enzyme (one-eighth and one-quarter respectively) and established that the yeast enzyme is composed of at least eight subunits of similar molecular weight. The studies were independently confirmed using

Table 2. Molecular parameters of yeast glutamine synthetase

Source and treatment of enzyme	Sedimentation coefficient ($\times 10^{13}$ s)	Stokes's radius (nm)	Apparent molecular weight	Frictional ratio	Synthetase to transferase activity ratio	pH optimum of enzyme
Crude extracts*	15.4	6.23	394 000	1.29	0.17	6.5
Purified enzyme	15.4	6.23	394 000	1.29	0.17	6.5
Urea dissociation of purified enzyme†	14.2	6.23	363 000§	1.32	0.17	6.5
	8.7	5.04	180 400	1.35	0.15	6.5
	5.8‡	—	90 700	—	—	—
	3.8‡	—	45 500	—	—	—
'Deactivated' enzyme	14.2	6.23	363 000	1.32	0.17	6.5
preparations‡	8.7	5.04	180 400	1.35	0.08	5.8
	5.8	—	90 700	—	—	—
	3.8	—	45 500	—	—	—

The figures are average values taken from at least three separate experiments.

* Crude enzyme extracts were obtained from yeast grown on aspartate, glutamate, alanine, ammonia and glutamine (all 10 mM) as sole sources of nitrogen. In all instances there were traces of activity (about 5 %) in a position corresponding to a 9 s component.

† The enzyme was pre-dissociated with 0.05 M-tris-urea (pH 9.6). Similar results were obtained when 0.03 M-sodium dodecyl sulphate was used.

‡ Deactivated enzyme was obtained by transferring glutamate grown yeast to glutamine (10 mM) for 1 h. Identical results were obtained when the enzyme was deactivated by transferring a culture to NH_4^+ (5 mM) or to a medium without a source of carbon.

§ The observed decrease in apparent molecular weight could be accounted for by an increase in $\bar{V}$ of the protein from 0.721 to 0.743 cm^3/g .

^{14}C -labelled enzyme; no evidence was obtained for the existence of any subunits of a molecular weight below 45 000, and hence we believe the yeast glutamine synthetase is an octameric globular protein.

The in vivo inactivation of glutamine synthetase

When cultures of *Candida utilis*, growing on glutamate as a nitrogen source, were transferred to a medium containing glutamine, deactivation of glutamine synthetase was observed; resuspension of the culture into glutamate led to a rapid restoration of the level of enzyme (Ferguson & Sims, 1971). In a similar experiment, enzyme extracts prepared at different times during enzyme deactivation and reactivation were examined on sucrose gradients and showed that increasing amounts of a molecular species with a sedimentation coefficient of about 8.7 s were formed (Fig. 4). As the synthetase to transferase ratio (0.08) and pH optimum of the enzyme (pH 5.8) distinguishes this component from the parent protein and from the tetramer produced by dissociating the enzyme with urea or sodium dodecyl sulphate (see Table 2), *in vivo* deactivation of glutamine synthetase must involve the dissociation of the active enzyme: the 8.7 s component which accumulates during enzyme deactivation, disappears during enzyme reactivation (Fig. 5). This component, however, was not observed in any appreciable quantity in yeast growing on a wide range of nitrogen sources. The molecular weight data confirm that it could arise from a halving of the parent protein. This idea is reinforced by a preparation of 8.7 s component, free of high molecular weight enzyme, spontaneously giving rise to an enzyme with sedimentation and synthetase transferase characteristics similar to the original enzyme (Fig. 6). The experiments described earlier, where highly purified ^{14}C -labelled enzyme was dissociated on urea gradients, establish that the specific transferase activity of the 8.7 s component was almost exactly one-half that of the native enzyme (Fig. 1c). Thus if we assume a synthetase to transferase ratio of 0.08 for the tetramer produced *in vivo*, the synthetase activity of this tetramer can, at most, be only one-tenth that

Fig. 3. Inactivation of glutamine synthetase. (a) Inactivation with alkali urea and protection by glutamate + ATP. Purified enzyme was incubated in 0.05 M-tris (pH 9.6) at 30 °C with different combinations of effectors and the activity decrement was followed by testing samples of the mixtures by transferase assay. System alone (●) (1 or 2 M urea in tris-HCl, pH 7.5, gave identical results); with 1 M-urea (○); with 1 M-urea + 60 mM-glutamate + 5 mM-ATP (■); with 1 M-urea + 5 mM-ATP; (▲) and with 1 M-urea + 10 mM-Mg²⁺ (□).

(b) Inactivation with sodium dodecyl sulphate (SDS). In similar experiments enzyme was incubated in 0.05 M-tris-HCl (pH 7.5) buffer alone (●); plus 0.03 % SDS (○); plus 0.06 % SDS (△); plus 0.03 % SDS + 10 mM-Mg²⁺ (□); and this 0.03 % SDS + 60 mM-glutamate + 5 mM-ATP (▲).

of the active enzyme. In some experiments synthetase transferase ratios as low as 0.03 were noted and therefore the tetramer produced *in vivo* is possibly without biosynthetic activity.

The small but progressive alteration in the sedimentation characteristics of parent enzyme during *in vivo* enzyme inactivation (Fig. 5) was confirmed by recentrifuging the enzyme taken from the sucrose gradient against marker proteins, which indicated that the reduction

Fig. 4. Changes in the sedimentation behaviour and activity characteristics of glutamine synthetase during the *in vivo* deactivation and reactivation of the enzyme. Enzyme deactivation was followed by transferring yeast grown on glutamate (6 mM) to a medium containing NH_4^+ (6 mM), and enzyme reactivation by transferring the yeast back to glutamate. Fractions were analysed for transferase activity at pH 6.5 (▲) and pH 5.8 (○) and for synthetase activity (□). Four times as much enzyme was used in the synthetase assays as in the transferase assays.

in the sedimentation of the protein was not because of the presence of the 8.7 *s* component in the extract. Protein-protein interactions between the two enzyme forms might conceivably have caused this retardation of the enzyme in the sucrose gradient.

The Stokes's radii of the 14.2 and 15.4 *s* components of yeast enzyme (Table 2) are identical within the limits set by the resolution of the agarose gel. Thus if the molecular weight of the protein is changed rather than its partial specific volume, the small difference in sedimentation coefficient represents removal of only about 25 amino acid residues per enzyme subunit. Although processes involving the removal of a few amino acid residues from a protein are not normally reversible, several independent lines of evidence suggest that this transition may be freely reversible. During enzyme purification, precipitation of deactivated enzyme (14.2 *s*) preparations with $(\text{NH}_4)_2\text{SO}_4$ yielded a protein with identical sedimentation, activity and chromatographic characteristics to the native (15.4 *s*) enzyme. When purified enzyme (15.4 *s*) was stored at -20°C with 1 M-glutamine, a decrease of the sedimentation coefficient to 14.2 *s* was observed without loss of catalytic activity; incubation of the enzyme with

Fig. 5

Fig. 6

Fig. 5. Changes in the amounts, and the sedimentation coefficient, of the octameric and tetrameric forms of glutamine synthetase during enzyme deactivation and reactivation. Procedure was as described in Fig. 4. (a) Changes in the amount of octamer (▲) and tetramer (●); (b) changes in the sedimentation coefficient of the octamer.

Fig. 6. Determination of the sedimentation coefficients of the various molecular conformations of glutamine synthetase. (a) Sucrose density centrifugation of the high molecular weight forms of the enzyme. Purified enzyme (Δ), deactivated enzyme (▲), and marker enzymes (○). The 14.2 s component used in this experiment was obtained by centrifuging a crude deactivated enzyme extract: fractions containing this activity were freed of sucrose and concentrated by ultrafiltration. (b) A crude deactivated enzyme preparation was centrifuged on a sucrose gradient. Fractions were assayed for transferase activity (▲) and synthetase activity (□). Marker enzymes were included (○). (c) Re-centrifugation of the enzyme tetramer. Tetramers isolated from the experiment above were used after they were desalted and concentrated by ultrafiltration. Fractions were assayed for transferase activity (▲) and synthetase activity (□). Four times as much enzyme was used in the synthetase assay as in the transferase assay in all these experiments.

60 mM-glutamate resulted in the restoration of the sedimentation coefficient. A similar reduction in sedimentation coefficient was found when purified enzyme was centrifuged on a gradient containing 1 M-urea (Fig. 1c). Comparable observations have been made with enzyme taken from the living organism. Measurements of the sedimentation coefficient of yeast enzyme during enzyme inactivation indicated that the sedimentation coefficient fell

Fig. 7. Sedimentation of glutamine synthetase monomers in a sucrose gradient in the presence or absence of glutamate or glutamate + Mg^{2+} . Monomers were obtained from deactivated enzyme preparations: fractions containing activity corresponding to sedimentation coefficients between 2 and 3 s were combined and concentrated by ultrafiltration. (a) The centrifugation of monomers in sucrose gradients or in ones containing glutamate and/or Mg^{2+} . Transferase activity profile from gradients containing 100 mM-glutamate + 1 mM- Mg^{2+} (▲), 100 mM-glutamate (■), sucrose control (●). (b) The transferase activity (▲) and synthetase activity (□) of oligomers arising from the re-association of enzyme monomers. Two marker proteins (○) were added to each gradient for calibration. The shape of the 21 s component was reproducibly 'skewed' and could contain a mixture of components with lower sedimentation coefficients.

progressively to about 14.2; when enzyme was reactivated *in vivo* by transferring the yeast back to glutamate its sedimentation coefficient was restored to 15.4 *s* within a few minutes, during which time there was very little increase in enzyme amount (Fig. 5).

When crude extracts containing deactivated enzyme were fractionated on sucrose gradients, lower molecular weight subunits were recognized in the resulting activity profile which resembled those observed when the enzyme was dissociated with urea. Components with sedimentation coefficients of 3.8 *s* (monomer), 5.8 *s* (dimer) and also 8.9 *s* (tetramer) were recognized. Similar results were obtained with preparations derived from yeast that had been transferred to a medium containing glutamine, or to a medium lacking in sources of carbon and nitrogen (Ferguson & Sims, 1974*a*). Evidently, in all these instances enzyme deactivation results from the dissociation of the native octamer, via its half enzyme, into subunits with very reduced catalytic activity.

As the half enzyme could spontaneously reassociate and give rise to a protein with similar characteristics to the parent enzyme, we investigated whether monomers could reversibly reassociate and form native enzyme. Monomers, essentially free of dimers, were prepared by fractionating crude, deactivated enzyme extracts on sucrose gradients: the appropriate fractions were desalted, concentrated, and layered on sucrose gradients and others containing 1 mM-Mg²⁺ and 1 mM-Mg²⁺ plus 100 mM-glutamate (Fig. 7). Only in gradients containing glutamate and/or magnesium were peaks of transferase activity seen in positions corresponding to about 15 *s* and 21 *s*. Although in both these instances appreciable activity was observed throughout the entire gradient, significantly more 21 *s* component was formed when both glutamate and magnesium were present. Extensive investigation of crude active and deactivated enzyme preparations has failed to reveal the presence of this 21 *s* component, which suggests that its formation is very much a feature of the *in vivo* conditions employed here. A calculation of its approximate molecular weight from the sedimentation data indicates that it is likely to be made up of about 15 or 16 subunits. In a repeat experiment, the activity profiles of Mg²⁺ and glutamate gradients were examined using synthetase and transferase assays. Although in this instance the majority of the activity was recovered as the 21 *s* component, there were clear indications of peaks at 7.6 as well as 3.8 *s*. This 7.6 *s* component probably corresponds to a trimer species. Observations of the synthetase activity profile indicate that the 21 *s* and 15 *s* components formed from the re-aggregation of monomers are catalytically inactive; the only synthetase activity to be observed was associated with 3.8 and 7.6 *s* components. In sucrose gradients a more even distribution of transferase activity was found; a 21 *s* component but no 15 *s* component was recognized. In none of these experiments was there any evidence that a tetramer could be re-formed from the monomeric subunit.

DISCUSSION

From hydrodynamic data, yeast glutamine synthetase has a molecular weight of approximately 395 000. Four of its subunits appear to be more tightly aggregated so that the enzyme consists of two loosely bound half molecules held together mainly by electrostatic and hydrogen bonds. Studies carried out *in vivo* indicate that deactivation involves several distinct stages. The first recognizable step is a freely reversible conformational change whereby the active enzyme is converted to its 'relaxed' form; the enzyme in this conformation is now seemingly particularly susceptible to dissociation. These results are reminiscent of the taut-relaxed transition observed with glutamine synthetase from *Escherichia coli* and where the relaxed form is also sensitive to dissociation (Shapiro & Ginsburg, 1968). Although the conditions needed to promote this conformational change within the yeast are not known at

present, the observation that active enzyme when freeze-thawed with glutamine can be converted to this form supports the idea that a build-up of a soluble pool of glutamine in the organism could be one factor responsible for initiating enzyme deactivation.

Analyses on deactivated enzyme extracts indicate that the next step in the deactivation process involves the separation of the two loosely-bound half molecules of the enzyme. Some evidence suggests that the tetramer produced *in vitro* differs from that formed by dissociation of native enzyme with urea; the former enzyme has altered pH activity characteristics and is almost completely devoid of synthetase activity. However, on standing, it frequently acquires the kinetic characteristics of the tetramer produced in urea. The changes in the enzyme structure associated with 15.4 to 14.2 *s* transition could involve reversible alterations to both the configuration of the individual subunits making up the half protein and the spatial relationship between the two half molecules. This second step in the deactivation process is also freely reversible *in vitro* and tetramers reassociate spontaneously to produce an enzyme with similar characteristics (pH optimum, synthetase transferase ratio and sedimentation coefficient) to the parent enzyme. Further stages in the deactivation of yeast glutamine synthetase include the dissociation of the tightly aggregated half molecule.

Incubation of enzyme monomers with magnesium ions and glutamate produced molecular aggregates with sedimentation coefficients of about 15 and 21 *s* suggesting, unexpectedly, that complete reaggregation of the fully dissociated subunits accompanied by a restoration of catalytic activity may be possible. We have shown elsewhere (Ferguson & Sims, 1974*b*) that the re-appearance of catalytic activity in the living yeast is very largely dependent upon *de novo* protein synthesis.

The precise reason why glutamine synthetase appears to re-aggregate in significant amounts *in vitro* and form enzyme with appreciable transferase activity and yet fail to reactivate in the living cell is not yet understood. Monomers do reassociate to produce higher aggregates with modified catalytic properties, indicating that at least some of the information necessary to reform native enzyme is present within the individual monomer. Aggregates with a greater number of subunits than the native protein are also formed under these conditions, suggesting that factors that normally operate to limit subunit bonding *in vivo* do not necessarily operate outside the cell. Intensive investigation has failed to reveal the presence of any 21 *s* component in cell-free extracts. As the 21 *s* component was only produced when trimer species were present this species might arise from a repeated stacking of trimers rather than from an association of tetramers. Glutamine synthetase from a number of other sources can undergo repeated subunit stacking following changes in the conformation of the native enzyme. Enzyme from pig brain (Stahl & Jaenicke, 1972) and chicken neural retina (Sarker, Fischman, Goldwasser & Moscona, 1972), can form helical aggregates, whilst the addition of Mn^{2+} or Co^{2+} to 'relaxed' enzyme from *Escherichia coli* can lead to the formation of long tubes resulting from a linear polymerization of hexamers (Valentine, Shapiro & Stadtman, 1970; see also Willms *et al.* 1967). In all these instances new binding sites form on the outer region of the protein molecule which permit a second type of isologous association to occur whereby patterns of subunit stacking, normally closed, become open.

An explanation for the difference in the *in vivo* and *in vitro* behaviour of the enzyme is that the chemical environment necessary to produce the physiologically active, tight-bound half enzyme is only found at or near the sites of protein synthesis. If, after transport of the enzyme to the site where it functions, the half enzyme undergoes dissociation, the individual subunits may not be able to assume the correct tertiary structure to form normal 'tight' tetramers.

The striking similarity in the structure of the mammalian enzyme (Wilk, Meister &

Haschemeyer, 1969; Tiemeier & Milman, 1972; Stahl & Jaenicke, 1972) and yeast enzyme raises the question as to whether the two systems are regulated in a similar way. Tiemeier & Milman (1972) have pointed out that the results of a number of *in vitro* studies with purified sheep brain, rat liver and Chinese hamster liver glutamine synthetase have not provided convincing evidence that the enzyme is subject to direct metabolic control, and they suggest that perhaps a system of control different from the cumulative feedback inhibition described in bacteria may operate in mammals. Their observations that glutamine is both a co-repressor of enzyme synthesis and that it can effect the rapid deactivation of the enzyme under some conditions indicates that the mammalian system has many features in common with food yeast (see Ferguson & Sims, 1974*a*). The mode of control emerging for glutamine synthetase in yeast may well be common to all eucaryotes.

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