COW BRAIN GLUTAMINASE: PURIFICATION
AND INFLUENCE OF PHOSPHATE AND BORATE
IONS ON ITS ACTIVITY AND MOLECULAR
WEIGHT
by
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Mahnaz Badamchian
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ABSTRACT

Cow Brain Glutaminase: Purification, and Influence of Phosphate and Borate on Its Activity and Molecular Weight

by

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An efficient and simple purification procedure for cow brain glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) is described. The main steps consisted of acetone extraction and French press treatment in the presence of phosphate ions, precipitation of nucleic acids and lipids by 0.08% protamine·SO₄ and high speed centrifugation (300,000xg), ammonium sulfate fractionation, and gel filtration on Sepharose 4B first as the low molecular weight and then as the aggregated form. The yield was 22% and the final preparation had a specific activity of 142 µmoles/min/mg. The purification was more than 8000-fold over crude brain homogenate. The enzyme showed one strong and one diffuse band on SDS gel electrophoresis, suggesting that the enzyme was highly purified.
Phosphate activated cow brain glutaminase with a sigmoidal concentration dependence. The activation was time dependent, a function of the pretreatment, and was enhanced by high concentrations of protein.

The molecular weight of cow brain glutaminase also depended on the nature of the buffer in which it is dissolved. Gel filtration on Sepharose 4B was used to determine the molecular weight. Three forms of glutaminase were observed, one each in tris/acetate, phosphate, and borate/phosphate buffer. These were interconvertible and have molecular weights of 170,000, 300,000, and >10^6 respectively. It appeared that activation of cow brain glutaminase by phosphate was due to formation of dimers or higher molecular weight polymers.

(94 pages)
INTRODUCTION

Glutaminase (EC 3.5.1.2 L-glutamine amidohydrolase) catalyzes the hydrolysis of the amide linkage in glutamine to produce ammonia and glutamate:

Glutamine + H$_2$O $\rightarrow$ Glutamate + NH$^+$

Krebs (1935) showed that this enzyme was distinct from a second enzyme activity, now designated glutamine-α-ketoacid transaminase (Greenstein & Carter, 1947).

In the mitochondria, the amine nitrogen of glutamate can be released by glutamate dehydrogenase:

Glutamate + NAD$^+$ $\rightarrow$ α-ketoglutarate + NH$_3$ + NADH + H$^+$

The resulting α-ketoglutarate can either be oxidized to CO$_2$ or converted to glucose.

In addition, glutamate can be converted back to glutamine by glutamine synthetase:

Glutamate + NH$_3$ + ATP $\rightarrow$ Glutamine + ADP + Pi

In the absence of a control mechanism, glutaminase and glutamine synthetase would cycle uselessly producing only the hydrolysis of ATP (Figure 1).

Glutaminase has been purified to homogeneity from pig brain (Svenneby et al., 1973; Nimmo & Tipton, 1980), pig kidney (Kvamme et al., 1970), and rat kidney (Curthoys et al., 1976b). The yield from pig brain was very low, only
Figure 1. "Futile cycle" of amide synthesis and degradation.
0.4-0.7%. It has also been partially purified from cow brain (Chiu & Boeker, 1979). The kidney enzyme has been variously reported to be immunologically identical to that from pig brain (Curthoys & Oliver, 1973), and to be structurally different (Olsen et al., 1970 & 1973; Svenneby et al., 1973).

A regulatory function for glutaminase may be indicated by the sensitivity of its activity to a wide variety of endogenous and exogenous effectors. Glutaminase requires phosphate or a phosphate containing compound for activity (Errera & Greenstein, 1949; Weil-Malherbe, 1969; Tveit et al., 1970) and appears to undergo self-association in the presence of multivalent anions such as phosphate and borate (Svenneby, 1970; Kvamme et al., 1970; Curthoys et al., 1976a; Curthoys & Godfrey, 1976).

So far there is no efficient and reproducible purification procedure for brain glutaminase. The mechanism of the effect of phosphate on brain glutaminase activity and aggregation state is still ambiguous.

There were two objectives to this study. The first was to develop a simple, reproducible, and efficient purification procedure for brain glutaminase. The second was to determine the effect of small ions such as borate and phosphate on the activity and molecular weight of brain glutaminase.
Each step of the available purification procedure (Chiu, 1979; Chiu & Boeker, 1979) was evaluated systematically. The resulting procedure produced highly purified enzyme with 22% yield and specific activity of 142 µmoles/min/mg. The maximum value previously reported for the brain enzyme (Nimmo & Tipton, 1980) was 50 µmoles/min/mg with 0.4% yield; and for the kidney enzyme (Curthoys et al., 1976b), it was 320 µmoles/min/mg with 11% recovery.

The activation of glutaminase by phosphate has been studied with respect to time, phosphate, glutamine, and protein concentration. The molecular weight and activity in different ionic environments was also investigated with gel electrophoresis and gel filtration on Sepharose 4B. Activation by phosphate is sigmoidal, time dependent, and enhanced by high concentrations of protein. It appears that there is a correlation between the phosphate-dependent activation of glutaminase and an increase in its molecular weight.
Glutamine Metabolism in Mammals

Glutamine is of major metabolic significance not only because it is a building block of most proteins, but also because it serves as a source of nitrogen for a variety of important biosynthetic pathways (Figure 2).

In view of the metabolic significance of glutamine it is perhaps not surprising to find that glutamine is the amino acid present in highest concentration in mammalian blood. It is also present in relatively high concentrations in other mammalian tissues such as the heart and brain.

The metabolism of glutamine in brain can be considered from at least three points of view. The first one is simply the description of its synthetic and degradative reactions, which are not very different from those in other tissues. Secondly, one might consider the role of glutamine synthesis in the elimination of ammonia, which has a well known toxic effect on the nervous system. Thirdly, there is the relationship of glutamine to the specialized mechanisms responsible for particular functions of the brain, namely the initiation, propagation and transmission of nerve impulses in neuronal structures. Studies in a number of laboratories, beginning with the discovery by Waelsch et al. of glutamate-glutamine metabolic compartmentation in brain tissue (Waelsch, 1960; Lajtha
Figure 2. Summary of glutamine metabolism.
et al., 1960; Berl et al., 1961; Takagaki et al., 1961), have established that glutamine is an important metabolic precursor to the pools of two amino acids which play a fundamental role in the transmission of nerve impulses across synapses in the central nervous system, namely \(\gamma\)-aminobutyric acid (GABA\*) and glutamate (Bradford et al., 1978; Reubi et al., 1978; Tapia & Gonzalez, 1978).

While initiation and propagation of nerve impulse are electrical phenomena, the transmission of impulses is a chemical process. The origin of a nerve impulse arises as a consequence of depolarization or loss of a Na\(^+\) gradient across the post-synaptic membrane. Compounds such as glutamate are called excitatory transmitters because they bring about this depolarization (Bradford & Richards, 1976; Hamberger et al., 1978a; Nadler et al., 1976; Sandoval et al., 1978; Yamamoto & Malsui, 1976). On the other hand, compounds such as GABA are inhibitory transmitters because they bring about hyperpolarization (Curtis, 1975; Krnjevic, 1974).

Glutaminase is the enzyme most responsible for the hydrolytic cleavage of glutamine to glutamate and ammonia. Glutaminase and glutamine synthetase interconvert glutamine and glutamate. High glutaminase and low or no glutamine synthetase activities have been reported in synaptosomes.

\*Abbreviations used: GABA = \(\gamma\)-aminobutyric acid.

\(V_t\) = total gel bed volume, \(V_0\) = void volume, \(V_e\) = volume at which the protein elutes, \(K_{av}\) = fraction of the volume of the gel that is available for the substance.
whereas glial cells are claimed to have low glutaminase and high glutamine synthetase activities (Hamberger et al., 1978b; Weiler et al., 1979). In addition to the compartmentation of glutamine and glutamate in brain (Berl et al., 1976; Van den Berg & Garfinkel, 1971) the distribution of glutaminase and glutamine synthetase activities in neural cells has been considered as supporting a glutamine cycle (Van den Berg & Garfinkel, 1971; Balazs et al., 1973; Henn et al., 1974). According to this theory, glutamate and \( \gamma \)-aminobutyric acid are taken up by glial cells via a high affinity transport mechanism (Henn et al., 1974), converted to glutamine and transported to the neurons to serve as substrate for the glutaminase reaction. Moreover, the small glutamate and large glutamine pools are considered to be localized in glial cells. The high degree of localization of glutaminase in the nerve endings may be of physiological importance at the level of glutamatergic and GABAergic synapses, Figures 3 and 4 (Tapia, 1980).

Subcellular Location of Glutaminase

Katunuma et al. (1967) have demonstrated the existence and have separated two different isoenzymes of glutaminase in the kidney, liver, and brain of rat. One enzyme, which requires phosphate or a related compound for activity, is usually referred to as phosphate-dependent glutaminase. The second, which is not affected by phosphate, but is strongly activated by maleate is referred to as phosphate-
Figure 3. Metabolic-physiological relationships in an excitatory glutamatergic synapse. Glutamate synthesized from glucose, pyruvate or glutamine is released by depolarization in the presence of Ca$^{2+}$. After interaction with its postsynaptic receptor it is taken up by the nerve endings or the adjacent glial cells by a Na$^+$-dependent mechanism. The glutamate entering the glia is converted to glutamine by glutamine synthetase (GS) and glutamine is eventually transported to the presynaptic ending to serve as a precursor of the releasable glutamate (Hamberger et al., 1978a & 1979)
Figure 4. Metabolic-physiological relationships in an inhibitory GABAergic synapse. GABA is synthesized from two different pools of glutamate by glutamate decarboxylase (GAD). The pool of glutamate derived from glucose or pyruvate, or taken up by the terminal, gives rise to GABA that is preferentially released in a process coupled to its synthesis. This release occurs in the absence of depolarization (spontaneous, tonic release), and is mediated by a population of GAD that binds to the presynaptic membrane in the presence of Ca\(^{2+}\). The other pool of glutamate, derived from glutamine, is decarboxylated in the cytoplasm of the nerve ending and the GABA thus synthesized is preferentially released by depolarization, in the presence of Ca\(^{2+}\). After interaction with its postsynaptic receptor, GABA is taken up by the nerve ending or the adjacent glial cells by a Na\(^{+}\) and Cl\(^{-}\)-dependent mechanism. In glia, GABA is transformed into glutamine (GABA is transaminated with \(\alpha\)-ketoglutarate by GABA-transaminase to yield succinic semialdehyde and glutamate which is converted to glutamine by glutamine synthetase), and this glutamine is transported to the nerve ending and converted to glutamate by glutaminase. In the nerve ending the GABA taken up may be metabolized by GABA-transaminase (Tapia, 1974; Tapia et al., 1975; Covarrullias & Tapia, 1978).
Glucose
  ↓
Pyruvate
  ↓
Citric Acid Cycle
  ↓
Glutamate
  ↓
Ca++ GAD
  ↓
GABA
  ↓
Receptor
  ↓
POSTSYNAPTIC NEURON

α-KG - α-ketoglutarate
GABA - γ-aminobutyric acid
GABA-T - GABA-transaminase
GAD - L-glutamate decarboxylase
GS - glutamine synthetase
SSA - succinic semialdehyde
independent glutaminase. In fact, the phosphate-independent glutaminase has since been shown to be a -transpeptidase (Tate & Meister, 1974 & 1975). Maleate promotes glutaminase activity while blocking trans-peptidation (Thompson & Meister, 1979).

The subcellular localization of the isoenzymes of phosphate-dependent glutaminase has been studied in rat kidney (Curthoys & Oliver, 1973; Curthoys & Weiss, 1974). It appears that glutaminase is contained in the inner mitochondrial membrane. During fractionation, glutaminase remains closely associated with cytochrome oxidase, an inner membrane marker and is separated from the matrix and outer membrane enzymes, malate dehydrogenase and NADH cytochrome C reductase, respectively (Curthoys & Weiss, 1974). In addition, a 10-fold greater concentration of either Lubrol or Triton X-100 is required to solubilize 60-70% of the glutaminase from the mitochondria than is needed to release the same percent of the total malate dehydrogenase activity (Curthoys et al., 1976a).

Rat, pig, and guinea pig brain contain only a phosphate-dependent glutaminase (Weil-Malherbe 1969; Weil-Malherbe & Beall, 1970; Huang & Knox, 1974). Pig brain mitochondria have been shown to contain two major forms of phosphate-dependent glutaminase; a readily extractable soluble enzyme located in the matrix and a membrane-bound enzyme located in the inner membrane. Only the latter
enzyme appears to be associated with mitochondria in nerve endings whereas both forms are present in free brain mitochondria (Nimmo & Tipton, 1979). An inner matrix location of glutaminase has also been reported from pig kidney (Chappell et al., 1972; Crompton et al., 1973) and rat kidney (Kalra & Brosnan, 1974) although an inner membrane location has also been reported for enzyme from the latter source (Curthoys & Weiss, 1974; Curthoys, 1974).

Kvamme & Olsen (1981), by using the sulfhydryl group reagent N-ethyl maleimide, reported that phosphate-dependent glutaminase in rat brain is localized in two distinct mitochondrial compartments within the inner mitochondrial membrane, and that phosphate dependent glutaminase of the external compartment is freely accessible to regulation by compounds in the cytosol such as hydrogen ion and phosphate. Since the Pi/OH-antiport which is responsible for the majority of phosphate transport is N-ethylmaleimide sensitive, Meijer et al. (1970) proposed that outer face glutaminase has a phosphate carrier function.

The physiological importance of the compartmentation of phosphate-dependent glutaminase is unknown. Kvamme & Olsen (1981) proposed that the fraction of glutamine hydrolyzed by phosphate-dependent glutaminase in the matrix is preferentially oxidized by the tricarboxylic acid cycle of the mitochondria and is, therefore, used for energy production. The fraction of glutamine that is hydrolyzed by
phosphate-dependent glutaminase on the outer face of the membrane may be used for production of glutamate which must be actively transported into the mitochondria to be oxidized. Therefore, Kvamme & Olsen (1981) proposed that its preferred use is to supply glutamate in the cytosol.

Purification of Glutaminase

In mammals, phosphate-dependent glutaminase is found principally in brain, kidney, and small intestine (Krebs, 1935; Errera & Greenstein, 1949; Horowitz & Knox, 1968). The function of this glutaminase appears to be different in each tissue.

Phosphate-dependent glutaminase was first solubilized from pig kidney (Otey et al., 1954), and partially purified by two different research groups (Klingman & Handler, 1958; Sayre & Roberts, 1958). Klingman & Handler (1958) purified the enzyme 200-fold by column chromatography on Amberlite, starting with a mitochondrial extract in borate buffer, whereas Sayre & Roberts (1958) obtained the same purification by sodium sulfate fractionation of kidney homogenates, which reportedly had to be kept frozen for several weeks. Kvamme et al. (1966) reported a method which allowed 1700-fold purification and which was based on the use of sodium sulfate precipitation followed by column chromatography on Amberlite. Later they reported a method which leads to about a 10,000-fold purification of pig kidney glutaminase with no detectable impurities (Kvamme
et al., 1970). The main steps consisted of sodium sulfate fractionation followed by alternative solubilization by dialysis against tris/Cl buffer and precipitation with phosphate/borate. Final specific activity was 320 µmoles/min/mg with a 3% yield.

Pig brain phosphate-dependent glutaminase has been purified to homogeneity by Svenneby et al. (1973), although in a very low yield (0.7%). This procedure consisted of the extraction of pig brain with acetone, followed by sodium sulfate fractionation of the solubilized acetone powder. Thereafter they used the tendency of the enzyme to aggregate in phosphate/borate buffer, a process which was reversed by transferring the enzyme to tris/Cl buffer, to purify the enzyme by a series of centrifugation steps.

Phosphate-dependent glutaminase has also been purified from rat kidney by Curthoys et al. (1976a) who also utilized the aggregation-disaggregation behavior in successive gel filtration steps. In this procedure the glutaminase was solubilized from the inner mitochondrial membranes by treatment with high concentrations of nonionic detergents or by lyophilization in the presence of borate, phosphate, and pyrophosphate ions. By subsequent gel filtration of the glutaminase in two different molecular weight forms, it was purified 1000-fold over crude kidney homogenates with a final specific activity of 320 µmoles/min/mg and an
11% yield. The purified glutaminase did not enter polyacrylamide gels in the absence of 0.1% Triton X-100.

The soluble phosphate-dependent glutaminase has been purified to homogeneity from pig brain (Nimmo & Tipton, 1980). This purification was based on a combination of the procedure used by Svenneby (1970) to purify glutaminase from pig brain and that devised by Curthoys et al. (1976a) to purify the enzyme from rat kidney. The final specific activity was 50 µmoles/min/mg with 0.4% yield.

Properties of Glutaminase

The glutaminase from kidney undergoes partial proteolysis when it is solubilized from lyophilized mitochondria (Clark & Curthoys, 1979). As a result, the purified enzyme consists of a mixture of structurally related peptides which range in molecular weight from 57,000 to 75,000. Brain enzyme does not appear to suffer proteolytic degradation. The molecular weight of the undegraded subunit is 82,000 (Clark & Curthoys, 1979; Chiu & Boeker, 1978).

The glutaminase from kidney and brain assumes three different states of aggregation, each having a different specific activity and depending, in a poorly understood way, on the nature of the dissolving buffer (Kvamme et al., 1966; Svenneby, 1970; Kvamme et al., 1970; Godfrey et al., 1977). If the enzyme is dialyzed against tris/Cl it has a molecular weight between 160,000 (Godfrey et al.,
1977) and 180,000 (Kvamme et al., 1970; Svenneby et al., 1973) as calculated from its Stokes radius and sedimentation coefficient. The tris form of glutaminase is inactive by itself; it is activated by phosphate and a number of other compounds such as carboxylic acids, riboflavin-5-P, ATP, acetyl-CoA, fatty acyl-CoA derivatives, thyroxine, bromothymol blue, borate, and sulfate (Van den Berg, 1973; Weil-Malherbe, 1969 & 1972a; Tveit et al., 1970; O'Donovan & Lotspeich, 1966; Kvamme et al., 1965a,b &1966; Kvamme & Torgner, 1974; Svenneby, 1970 & 1971; Kvamme, 1979; Svenneby et al., 1970). Although this activation is not completely understood, the kinetic data suggest that at least two binding sites each for phosphate and borate are involved (Svenneby, 1971; Kvamme & Torgner, 1974 & 1975; Nimmo & Tipton, 1981). Both phosphate (Godfrey et al., 1977; Svenneby, 1972) and acetyl-CoA (Kvamme & Torgner., 1974) are also known to cause polymerization of the tris form; for phosphate, this polymerization with kidney glutaminase results in a dimer with a molecular weight of 330,000 and is well correlated to the increase in activity (Godfrey et al., 1977). It appears that pig brain glutaminase does not undergo phosphate-stimulated dimerization (Svenneby, 1970). This phenomenon has not been investigated with the cow brain glutaminase. It is not altogether clear, however, that dimerization of the tris form is a necessary prerequisite for activity; a number of studies suggest that
allosteric activation and dimerization are separate processes (Svenneby, 1971; Kvamme & Torgner, 1974; Svenneby et al., 1970). Addition of borate ions to the phosphate form apparently causes a conformational change as well as further polymerization and produces a third form with a very high molecular weight (Kvamme et al., 1970; Olsen et al., 1973; Svenneby, 1970).
EXPERIMENTAL PROCEDURES

Materials

Bovine serum albumin, thyroglobulin, catalase, aldolase, ovalbumin, blue dextran 2000, and glutamate decarboxylase type I from *Escherichia coli* were purchased from Sigma. PM-30 ultrafiltration membranes were obtained from Amicon Co. Fresh cow brains were obtained from the E. A. Miller and Sons Packing Co., Hyrum, Utah. Sepharose 4B and 6B were purchased from Pharmacia. Omnifluor, \( L-(U^{14}C) \)-glutamine and \( L-(U^{14}C) \)-glutamic acid were obtained from New England Nuclear. Other reagents were analytical grade.

For the glutamate decarboxylase used in the glutaminase assay, 5 grams of *E. coli* acetone powder were suspended in 10 ml of 0.1 M K-phosphate, 1 mM dithiothreitol, and 1 mM pyridoxal phosphate (pH 4.2), and homogenized for 5 minutes in a Potter-Elvehjem homogenizer with a teflon pestle. The homogenate was used directly in the assay.

Several buffers are referred to repeatedly in this work. They are:

Barbital: 10 mM Na-barbital pH 8.6.

Borate: 20 mM Na borate, pH 8.8.

Borate/phosphate: 10 mM Na borate, 100 mM Na-pyrophosphate, and 100 mM K-phosphate pH 8.9.


Tris/acetate: 10 mM Trizma acetate, pH 8.6.
Tris/pyrophosphate: 10 mM tris/Cl, 100 mM Na-pyrophosphate, and 200 mM dithiothreitol, pH 8.8.
Tris/phosphate: 10 mM tris/acetate and 100 mM K-phosphate, pH 8.8.

Protein Determinations

Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard. In the presence of Triton X-100 the modified Lowry method (Chandra Rajan & Klein, 1975) was used. The concentration of purified glutaminase was estimated by measuring the absorbance at 280 nm and assuming that an absorbance of 1 corresponds to a protein concentration of 1 mg/ml (Warburg & Christian, 1941).

Glutaminase Assays

The assay procedure was based on the method of Chiu & Boeker (1979, see also Dinwoodie & Boeker, 1979) in which the glutamate produced by glutaminase is converted to $^{14}$CO$_2$ by *E. coli* glutamate decarboxylase.

To start the reaction, 100 µl of enzyme sample was added to 200 µl of substrate solution which contained 0.2 M L-[U-14C] glutamine and 0.2 M K-phosphate, pH 8.8, and had been equilibrated at 38°C for 5 minutes. After 10 minutes, 0.4 ml of 0.4 M pyridine - HCl, pH 1.3, was added to stop the reaction and reduce the pH to 4.6 ± 0.3. Decarboxylation was begun by adding 3 units of *E. coli* glutamate decarboxylase in 100 µl of 0.1 M KH$_2$PO$_4$, 1 mM
dithiothreitol, and 1 mM pyridoxal phosphate (pH 4.2). The
$^{14}\text{CO}_2$ was trapped on a filter paper soaked with 25 µl of
a 1:2 solution of 2-amino ethanol and 2-methoxyethanol and
placed in the neck of the test tube. After 60 minutes,
the filter paper was transferred to a vial containing 10 ml
of scintillant (15 g Omnifluor, 750 ml 2-methoxyethanol and
3 liters of toluene) and counted in Beckman LS 100 Scintil-
lation Counter.

The effective specific activity for glutamine was
measured experimentally by adding enough glutaminase to
exhaust the glutamine present. An exhaustion value was
determined for each series of assays. Samples were either
assayed directly or diluted with the sample buffer if
needed. Control assays were done by replacing the enzyme
solution with the sample buffer.

In order to check the validity of the assay, several
experiments were done. Figures 5 and 6 show that at least
60 minutes and 3 units of glutamate decarboxylase are
needed to decarboxylate all of the glutamate found in the
glutaminase assay. The commercially available glutamate
decarboxylases also decarboxylate glutamine to a small
extent. Figure 7 shows that, while the glutamate decarb-
oxylase used here does produce CO$_2$ from glutamine, the
amount is not significant (0.9% of exhaustion). For all
assays in this study the results were corrected by running
a control at each time point.
Figure 5. Conversion of glutamate to CO₂. 0.2 M glutamine was replaced with 0.2 M glutamate and incubated with 3 units glutamate decarboxylase under standard assay conditions.
Figure 6. A. Units of glutamate decarboxylase needed to decarboxylate all of the resulting glutamate from 0.2 M glutamine under standard assay conditions. B. Time needed to convert glutamate to CO₂ using 3 units glutamate decarboxylase under standard assay conditions. Five units of glutaminase were used in both A and B.
Figure 7. Incubation of 0.2 M glutamine with 3 units glutamate decarboxylase in the absence of glutaminase under otherwise standard assay conditions.
Figure 8A shows that the glutaminase assay is linear with time for 60 minutes. Therefore, long assay times were used in an attempt to determine very low specific activity. Figure 8B shows the linearity with respect to the amount of substrate consumed. The experimental curve begins to deviate from a straight line at 90% exhaustion. Assays in this study have been done at 70% exhaustion or less.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed according to the procedure of Davis (1964). Samples containing 20% glycerol or sucrose were run on 7.5% polyacrylamide tube gels at 3 mA/gel. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970). Gels were stained with 0.05% Coomassie Brilliant Blue in methanol: acetic acid:water, 5:1:5, and destained in 5% methanol:7.5% acetic acid.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed according to the procedure of Helling et al. (1974). Gels (6 x 0.6 cm) were formed in 7 cm glass tubing with nylon netting (panty hose) across one end to retain the gel. Agarose (LITEX) Type LSB 0.7-1.4% in tris-glycine buffer pH 8.0 (tris, 6 g; glycine, 29 g; H₂O to 1 L with or without 0.1% Triton X-100) was melted by heating. A small amount was used to
Figure 8. A. Linearity of standard assay with time. 0.2 M glutamine incubated with 5 units of glutaminase for different lengths of time under standard assay conditions. B. Percentage exhaustion that is linear in assay. 0.2 M glutamine incubated with different concentrations of glutaminase for ten minutes using standard assay conditions.
seal the bottom of the tube; then additional agarose (cooled below 60°C) was added to fill the column. After hardening, the upper end of the gel was extruded and sliced evenly to form a 6 cm gel. Samples containing Bromophenol blue and glycerol (20%) were run into the gel for 5 minutes at 100 v and thereafter at 1.5 v/cm of gel. Gels were stained with 0.05% Coomassie Brilliant Blue in methanol: acetic acid:water, 5:1:5, and destained in 5% methanol: 7.5% acetic acid.

Molecular Weight Determinations

The proteins used as standards, and their sources, are shown in Table 1.

The Sepharose was washed with four volumes of water followed by several washings of 1 L volumes each with equilibration buffer. The gel was then poured into a 60 x 0.8 cm Pharmacia column and allowed to settle under gravity. Final equilibration was achieved by running 2-3 volumes of

<table>
<thead>
<tr>
<th>Table 1. Proteins Used as Standards.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>--------------------------------------</td>
</tr>
<tr>
<td>Aldolase</td>
</tr>
<tr>
<td>Catalase</td>
</tr>
<tr>
<td>Ferritin</td>
</tr>
<tr>
<td>Thyroglobulin</td>
</tr>
</tbody>
</table>

*Molecular weights and Stoke's radii are those given in the Gel Filtration Calibration Kit Instruction Manual, Pharmacia Fine Chemicals, Piscataway, New Jersey 08854.
the buffer through the column. The column was run at a flow rate of 5 ml/hour. A pressure drop of approximately 30 cm was required to maintain this flow rate.

The proteins were dissolved in the column solvent (0.5-5 mg of each) and were run through the column individually as well as in a mixture. The samples were applied on the top of the gel; the sample size was 1.2% of the total gel bed volume ($V_t$). Fractions of 0.5-1 ml were collected and monitored by measuring absorbance at 280 nm. Blue dextran was routinely run through the column to determine the void volume ($V_0$). All chromatographic experiments were carried out at 4°C.

The fraction of the volume of the gel that is available for each protein ($K_{av}$) was calculated by the using the equation (Laurent & Killander, 1964) $K_{av} = (V_e - V_0) / (V_t - V_0)$, where $V_e$ is the volume at which the protein elutes. A linear relationship exists between the $K_{av}$ value for each standard protein and the logarithm of the corresponding molecular weights.
RESULTS

Glutaminase Purification: Preliminary

The method developed for the purification of cow brain glutaminase was a modification of the method described earlier by Chiu (1979). This purification was based on a combination of the procedure used by Svenneby et al. (1973) to purify a phosphate-activated glutaminase from pig brain, and that devised by Curthoys et al. (1976a) to purify the enzyme from rat kidney. Chiu's procedure consisted of the preparation of an acetone powder from frozen brain; homogenization in 20 mM Na-borate, pH 8.8, (1:20, w/v) for two minutes; precipitation of nucleic acids by 0.01% protamine-SO₄ and 10% (NH₄)₂SO₄; 23% (NH₄)₂SO₄ fractionation; a batchwise calcium phosphate gel step; and gel filtration on Sepharose 4B, first as the aggregate and then as the low molecular weight form.

Figures 9 and 10 show the gel filtration of glutaminase on two Sepharose 4B columns in an early attempt to reproduce Chiu's work. As can be seen, the activity tended to distribute itself throughout the entire elution volume of the first column (Figure 9). Furthermore, the second column did not differentiate the activity from the impurities (Figure 10). Other experiments also indicated that the extraction conditions and (NH₄)₂SO₄ fractionation used by Chiu were not entirely optimal. A systematic evaluation of the purification procedure was therefore undertaken.
Figure 9. Gel filtration of glutaminase in borate/phosphate buffer on Sepharose 4B. Glutaminase (25 ml of a 94 mg/ml solution) prepared according to Chiu (1979) was applied to a 4.2 x 60 cm column equilibrated with 10 mM sodium borate, 100 mM sodium pyrophosphate, and 100 mM potassium phosphate buffer, pH 8.9. The fractions between the arrows were pooled and used for the column shown in Figure 10.
Figure 10. Gel filtration of glutaminase in tris/pyrophosphate buffer on Sepharose 4B. The column (2.6 x 100 cm) was equilibrated in 10 mM tris-Cl, 100 mM sodium pyrophosphate, and 0.2 mM dithiothreitol buffer, pH 8.6. Glutaminase (13 ml of a 13.4 mg/ml solution) obtained as shown in Figure 9 was dialyzed against this buffer and applied to the column.
Glutaminase Purification: Extraction

As described by Chiu, two hundred grams of acetone powder were suspended in 4 liters (1:20, w/v) of 20 mM sodium borate and 2 mM EDTA (pH 8.6), homogenized at 240 rpm in a Potter-Elvehjem homogenizer with a teflon pestle for two minutes, and centrifuged at 16,000xg for 20 minutes at 0°C. Figures 11 and 12 show that increasing the time or the volume of the homogenization step increased the amount glutaminase extracted. Figure 13 shows that incubation of the homogenate for 4 hours at 4°C doubled the amount of activity released. Figure 12 also shows that, while no glutaminase activity could be detected in the precipitate after the homogenate was centrifuged, further homogenization increased the activity released.

These results indicated that a stronger method was required to release glutaminase from the acetone powder. In the results shown in Table 2, 16 grams of acetone powder were suspended in 640 ml (1:40, w/v) of 0.2 M potassium phosphate buffer, pH 8.8, 1 mM EDTA, 0.05% (v/v) 2-mercaptoethanol and passed through a French pressure cell at 20,000 psi. This extraction procedure was adopted for further use.

Chiu used a 20 mM MgCl₂, 0.01% protamine sulfate, and a 10% (NH₄)₂SO₄ precipitation to remove nucleic acids and other impurities from the glutaminase supernatant after homogenization. Glutaminase was then precipitated with 23%
Figure 11. Effect of buffer volume on the homogenization step. One gram of acetone powder was suspended in borate/phosphate buffer (pH 8.9), and homogenized at 240 rpm in Potter-Elvehjem homogenizer with teflon pestle for 5 minutes.
Figure 12. Effect of time on homogenization step. One gram of acetone powder was suspended in 40 ml of borate/phosphate buffer and homogenized as indicated in Figure 11.
Figure 13. Release of glutaminase activity after homogenization. One gram of acetone powder was suspended in 40 ml borate/phosphate buffer (pH 8.9, homogenized 2 minutes, and incubated at 4°C before being centrifuged.
Table 2. Effect of Different Treatments of the Acetone Powder on the Release of Glutaminase.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (min)</th>
<th>w/v (g/ml) acetone powder</th>
<th>Units/g acetone powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - Homogenization</td>
<td>2</td>
<td>1/20</td>
<td>15</td>
</tr>
<tr>
<td>2 - Homogenization*</td>
<td>10</td>
<td>1/40</td>
<td>60</td>
</tr>
<tr>
<td>3 - 2nd Homogenization**</td>
<td>10</td>
<td>1/12</td>
<td>25</td>
</tr>
<tr>
<td>4 - French press</td>
<td>-</td>
<td>1/40</td>
<td>100</td>
</tr>
</tbody>
</table>

*16 g of acetone powder was suspended in 640 ml of 0.2 M potassium phosphate buffer, pH 8.8, 1 mM EDTA, 0.05% (v/v) 2-mercaptoethanol, homogenized at 240 rpm in a Potter-Elvehjem homogenizer with a teflon pestle.
**Further homogenization of the precipitate after the homogenate in 2 was centrifuged.

(NH₄)₂SO₄, centrifuged, and resuspended in 20 mM sodium borate and 2 mM EDTA. Calcium phosphate gel (1:1, w/v) was added to this suspension in order to remove additional nucleic acids and decrease the viscosity of the sample. According to Chiu glutaminase binds to the calcium phosphate in the absence of phosphate, and is released by 0.2 M phosphate. "Without this treatment the sample sticks to the top of chromatographic columns, making separation impossible."

In this study the amount of protamine sulfate was increased to 0.08%, increasing the 280/260 ratio dramatically. The supernatant after the 10% (NH₄)₂SO₄ precipitation was turbid, suggesting that lipids were still
present. Addition of a 300,000xg centrifugation before 
(NH$_4$)$_2$SO$_4$ fractionation produced a clear supernatant. The 
enzyme then precipitated with 40% (NH$_4$)$_2$SO$_4$. When redis­solved in 0.2 M phosphate buffer, a clear preparation was 
obtained which chromatographed properly on a Sepharose 4B 
column.

In addition, it appeared that glutaminase did not bind 
to the calcium phosphate gel completely in the absence of 
phosphate. Since centrifugation clarified the preparation, 
this step was omitted, also alleviating the need to start 
the purification in the absence of phosphate, which de­
creased the yield of glutaminase.

In the modified procedure, the acetone powder was 
prepared as described by Chiu (1979), suspended in phosphate 
buffer and treated in a French pressure cell. The resulting 
suspension was centrifuged at 20,000xg for 30 minutes. 
Solid MgCl$_2$•6H$_2$O, 2.5 grams, and protamine-SO$_4$, 0.5 g were 
added to the supernatant solution. The resulting suspension 
was stirred slowly for one hour at 4°C and then centrifuged 
at 20,000xg for 30 minutes. The cloudy supernatant was 
centrifuged at 300,000xg for 2 hours. The clear supernatant 
was taken to 40% saturation with ammonium sulfate (24.3 
g/100 ml) and allowed to stand at 4°C for 30 minutes, then 
centrifuged at 20,000xg for 30 minutes. The precipitate 
(17.6 g) was suspended in 10-12 ml of 0.2 M K-phosphate 
buffer, pH 8.8, 1 mM EDTA, 0.05% (v/v) 2-mercaptoethanol
and dialyzed against two, 2 liter changes of the same buffer overnight. Any undissolved material was removed by centrifugation at 39,000xg for 30 minutes.

Glutaminase Purification: Sepharose 4B Columns

The specific activity and molecular weight of glutaminase are higher in borate/phosphate buffer (associated form) than in tris or phosphate buffers (lower molecular weight forms). This property was used by Svenneby (1970) to purify the pig brain enzyme by successive centrifugation steps. Curthoys et al. (1976a) used successive gel filtration steps to purify the rat kidney enzyme. Chiu (1979) also took advantage of this, using Sepharose 4B columns to differentiate glutaminase from impurities by chromatographing first with borate/phosphate buffer (associated form) and then with tris/pyrophosphate buffer (lower molecular weight form).

For gel filtration Chiu applied 25 ml of a 94 mg/ml sample on the first and 13 ml of a 13.4 mg/ml on the second Sepharose 4B column. Attempts to reproduce Chiu's elution profiles were not successful. Gel filtrations under these conditions did not differentiate the activity from the impurities (Figures 9 and 10). It seemed possible that decreasing the sample size, protein concentration, and column flow rate would improve the separation. Figure 14 shows the elution profile that results when a sample prepared using the modified extraction procedure just
Figure 14. Gel filtration of glutaminase in borate/phosphate buffer on Sepharose 4B. 25 ml of a 15 mg/ml sample was applied to a 4.5 x 90 cm Sepharose 4B column equilibrated with 10 mM Na-borate, 100 mM Na-pyrophosphate, and 100 mM K-phosphate buffer, pH 8.9. Glutaminase was eluted with same buffer at a flow rate of 10 ml/hour.
described was applied to a Sepharose column in borate/phosphate (associating) buffer and the sample size, protein concentration, and column flow rate were decreased to 2%, 15 mg/ml, and 10 ml/hour respectively. As can be seen, both bulk proteins and glutaminase were still eluted at the void volume.

In one attempt to improve the elution profiles of glutaminase, Triton X-100 was added to the extraction buffers, and the initial gel filtration of glutaminase was carried out in dissociating (tris/pyrophosphate) buffer plus Triton. The results were not promising. Although the glutaminase peak shifted so that it was now centered near 60% of the column volume, it still overlapped the bulk protein peak.

Figure 15 shows the behavior of glutaminase on Sepharose 4B in associating (borate/phosphate) buffer after extraction of the acetone powder with borate/phosphate buffer plus Triton. The fractions between the arrows were pooled and used for a Sepharose 4B column in the presence of dissociating buffer; this produced a good separation (Figure 16).

It is apparent from the column profiles shown in Figures 15 and 16 that in order to differentiate the glutaminase from impurities in the presence of Triton, the enzyme must be applied first as the aggregated form and second as the low molecular weight form.
Figure 15. Gel filtration of glutaminase on Sepharose 4B in the presence of Triton X-100. 80 ml of a 10 mg/ml sample was applied to a 4.5 x 90 cm column equilibrated with 10 mM Na-borate, 100 mM sodium pyrophosphate, 100 mM K-phosphate (borate/phosphate) and 2% Triton X-100 buffer, pH 9.9. Glutaminase was eluted with same buffer at a flow rate of 10 ml/hour. The fractions between the arrows were pooled and used for the Sepharose 4B column in Figure 16.
Figure 16. Gel filtration of glutaminase in tris/pyrophosphate buffer on Sepharose 4B. 20 ml of a 1.1 mg/ml sample was applied to a 2.6 x 100 cm Sepharose 4B column equilibrated with 10 mM Tris/Cl, 100 mM Na-pyrophosphate, and 0.2 mM dithiothreitol buffer, pH 8.6. Glutaminase was eluted with the same buffer at a flow rate of 15 ml/hour.
Although this approach seemed useful, there are a number of disadvantages to using Triton during the purification: (1) its high absorbancy at 280 nm interferes with protein estimates; (2) it precipitates during 40% ammonium sulfate fractionation; (3) enzyme prepared in its absence would be preferable for physico-chemical studies.

Although the bulk protein is retarded on Sepharose 4B in the presence of Triton, preliminary studies indicated that it eluted near the void volume in its absence. Therefore, 0.2 M phosphate (dissociating) buffer was used to extract the acetone powder, and glutaminase was chromatographed on the Sepharose 4B column in the presence of this buffer. As shown in Figure 17, it appeared at 50-70% of column volume, well retarded from the void volume, where the bulk protein eluted. When the fractions between the arrows were pooled and dialyzed against borate/phosphate buffer, glutaminase aggregated and was then eluted near the void volume of the second Sepharose 4B column as shown in Figure 18. The time of dialysis before chromatography was critical; a minimum of 30 hours were required to obtain complete aggregation. A column run after a shorter dialysis time showed some dissociated glutaminase at 50-70% of the column volume.

Figure 19 shows the calibration of Sepharose 4B columns which were used during purification. As can be seen with associating buffer (borate/phosphate), glutaminase
Figure 17. Gel filtration of glutaminase in K-phosphate buffer on Sepharose 4B. 30 ml of a 19 mg/ml sample was applied to a 4.5 x 90 cm Sepharose 4B column equilibrated with 200 mM K-phosphate buffer, pH 8.8, 1 mM EDTA, 0.05% (v/v) 2-mercaptoethanol. Glutaminase was eluted with the same buffer at a flow rate of 10 ml/hour.
Figure 18. Gel filtration of glutaminase in borate/phosphate buffer on Sepharose 4B. 30 ml of a 1.2 mg/ml sample was applied to a 2.6 x 100 cm Sepharose 4B column equilibrated with 10 mM Na-borate, 100 mM Na-pyrophosphate, 100 mM K-phosphate, 1 mM EDTA, and 0.05% (v/v) 2-mercaptoethanol, pH 8.8. Glutaminase was eluted with the same buffer at a flow rate of 10 ml/hour.
Figure 19. Gel filtration of glutaminase and globular proteins with known molecular weights on Sepharose 4B. A 4.5 x 90 or 2.6 x 100 column was equilibrated either with 200 mM K-phosphate buffer, pH 8.8 or with 10 mM Na-borate, 100 mM Na-pyrophosphate, and 100 mM K-phosphate buffer, pH 8.9. The molecular weights of the standard proteins are shown in Table 1. The void volume of the column was determined with blue dextran.
appeared near the void volume and overlapped the blue
dextran peak. With phosphate buffer, glutaminase appeared
between 50-70% of the column volume corresponding to a
molecular weight somewhat higher than 232,000, that of
catalase. The observed Kav values for the standard proteins
were almost the same in borate/phosphate and phosphate
buffers.

In the final version of the purification procedure,
the supernatant from the last step of the extraction was
applied to a 4.5 x 90 cm column of Sepharose 4B equilibrated
with 0.2 M K-phosphate buffer, pH 8.8, 1 mM EDTA, 0.05%
(v/v) 2-mercaptoethanol. Glutaminase eluted at 50-70% of
the column volume. The flow rate was 10 ml/hour. The
active fractions were pooled and concentrated to 10 ml in
an Amicon ultrafiltration cell using a Diaflo PM-30 mem-
brane. The sample was then dialyzed against two, 2 liter
changes of 0.1 M Na-borate, 0.1 M Na-pyrophosphate, 0.1 M
K-phosphate, 1 mM EDTA, and 0.05% (v/v) 2-mercaptoethanol,
ph 8.9 (borate/phosphate buffer + EDTA + 2-mercaptoethanol)
for at least 30 hours. Undissolved materials were removed
by centrifugation at 39,000xg for 30 minutes. The super-
natant was applied to a 2.6 x 100 cm column of Sepharose 4B
equilibrated in the above buffer. The glutaminase eluted
at the void volume of the column. The flow rate was 15
ml/hour. Appropriate fractions were pooled and concentrated
to 10 ml in an Amicon ultrafiltration cell as above. The
purified glutaminase, stored in borate/phosphate buffer at 0°C, was stable for several months.

Glutaminase Purification: Summary

The purification procedure presented in this work is summarized in Table 3. The overall yield was 22%; 100 of brain cerebrum gave 2.1 mg of glutaminase. This is higher than the yield obtained for the pig brain (Svenneby et al., 1973) and rat kidney (Curthoys et al., 1976a) enzymes. The addition of mercaptoethanol to the buffers used during purification improved the reproducibility of the procedure. The entire purification procedure could be completed in 4-5 days, the most time consuming steps being those involving gel filtration and dialysis. The final preparation has been purified more than 8000-fold with a specific activity of 142 µmoles/min/mg.

The specific activity of glutaminase depended on the composition of the buffer in which it was stored; the enzyme was more stable in borate/phosphate buffer. This will be described in detail in a later section.

Acrylamide Gel Electrophoresis

Figure 20 shows polyacrylamide gel electrophoresis (Davis, 1964) of cow brain glutaminase at different steps of the purification. Figure 21 A shows electrophoresis of purified glutaminase which had been dialyzed against different buffers. As can be seen, glutaminase has
<table>
<thead>
<tr>
<th>Step</th>
<th>Volume ml</th>
<th>Protein mg</th>
<th>Activity μmole/min</th>
<th>Sp. Act. μmole/min/mg</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract of acetone powder</td>
<td>640</td>
<td>12160</td>
<td>1920</td>
<td>0.16</td>
<td>100</td>
</tr>
<tr>
<td>300,000xg supernatant after protamine-SO₄</td>
<td>500</td>
<td>3400</td>
<td>1764</td>
<td>0.5</td>
<td>92</td>
</tr>
<tr>
<td>Supernatnat after 40% (NH₄)₂SO₄ and dialysis</td>
<td>30</td>
<td>570</td>
<td>1222</td>
<td>1.6</td>
<td>64</td>
</tr>
<tr>
<td>First sepharose 4B column, pooled fractions (after dialysis against borate/phosphate buffer)</td>
<td>30</td>
<td>36</td>
<td>873</td>
<td>24.4</td>
<td>45.5</td>
</tr>
<tr>
<td>Second sepharose 4B column, pooled fractions (in borate/phosphate buffer)</td>
<td>10</td>
<td>3</td>
<td>425</td>
<td>142</td>
<td>22</td>
</tr>
</tbody>
</table>

*Prepared from 143 g cerebrum containing as little as white matter as possible.*
Figure 20. Native polyacrylamide gel electrophoresis of cow brain glutaminase at different stages of the purification. Left to right: Acetone powder after French press treatment, supernatant after centrifugation at 20,000xg for 30 min, supernatant after centrifugation at 300,000xg for 2 hours, glutaminase before first Sepharose 4B column in phosphate buffer, glutaminase after first gel filtration in phosphate buffer, and glutaminase after 2nd gel filtration in borate/phosphate buffer. 40 µg of protein was applied in each case.
Figure 21. Polyacrylamide gels of purified glutaminase. A. The glutaminase was dialyzed against (left to right) borate/phosphate, phosphate, tris/acetate, or barbital buffer. B. SDS polyacrylamide gels of the top samples in the same order. 40 µg protein was applied in each case.
different electrophoretic properties depending on the buffer it has been dialyzed against: borate/phosphate, or phosphate, tris/acetate, or barbital. Borate/phosphate gives a protein band only at the top of the stacking gel. The other three buffers give two protein bands, one between the stacking and running gels and the other well down in the running gel. The activity of the glutaminase in these bands was determined as follows: gels were frozen and sliced crosswise, and the slices were eluted overnight at 4°C in 200 μl of borate/phosphate buffer. Aliquots of the eluted material were then assayed under standard conditions. All of the protein bands were active.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of the glutaminase samples shown in Figure 21 A gave, in each case, one strong and one diffuse band, as shown in Figure 21 B. The subunit molecular weight of the strong band was estimated from a calibration curve using ovalbumin, bovine serum albumin, aldolase, and catalase, and found to be approximately 80,000. This value is similar to that found by Chiu (82,000 ± 2,000).

In an effort to get the aggregated form of glutaminase to enter the gel during electrophoresis, the following methods have been tried: (a) using 0.7 or 1.4% agarose gel in the presence or absence of 0.1% Triton X-100, (b) carrying out electrophoresis at pH 4.3 (Reisfeld et al.,
1962). Both techniques are described in "Experimental Procedure." Neither approach was successful.

When polyacrylamide gel electrophoresis of the dissociated form of glutaminase was carried out at pH 4.3 and compared with that at pH 8.3 (Davis, 1964), it appeared that this form was positively charged at pH 4.3 and negatively charged at pH 8.3, as is to be expected for a normal globular protein.

Activation of Glutaminase

The effect of different buffers on the stability of glutaminase is shown in Figure 22. The enzyme is stable when stored in borate/phosphate buffer and unstable when stored in tris/acetate or barbital buffer. It is reasonably, but not completely, stable when stored in tris/acetate plus phosphate. In control experiments not shown, the effect of these buffers on the glutamate decarboxylase in the standard glutaminase assay was tested; no significant differences in the release of CO₂ were detected.

Cow brain glutaminase required phosphate for its activity and was completely inactive in its absence. The activation of glutaminase that had been dialyzed against tris/acetate buffer is shown for different phosphate concentrations in Figure 23. Figure 24 shows the activation of glutaminase by high concentrations (200 mM) of phosphate at 38°C during the assay. Enzyme that had been preincubated in borate/phosphate (○, ●, ▲) showed a normal release of
Figure 22. Stability of glutaminase exposed to different buffers at 4°C. Glutaminase (1.5 mg/ml) was dialyzed against borate/phosphate buffer, pH 8.9 (•), borate buffer, pH 8.8 (●), phosphate buffer, pH 8.8 (◆), tris/acetate + phosphate buffer, pH 8.8 (◇), barbital buffer, pH 8.6 (○), or tris/acetate buffer, pH 8.6 (○) and assayed under standard conditions.
Figure 23. Activation of glutaminase by phosphate; pre-incubation in tris/acetate. Glutaminase (1 mg/ml, in borate/phosphate buffer) was dialyzed for 4 hours against tris/acetate buffer and then incubated and assayed in the presence of varying levels of phosphate under otherwise standard assay conditions. The activity of enzyme in borate/phosphate buffer assayed under standard assay condition was assumed to be 100%.
Figure 24. Time dependent activation of glutaminase by phosphate; preincubation in borate/phosphate, phosphate, and tris/acetate. Glutaminase (1.2 mg/ml) dialyzed for 4 hours against borate/phosphate (○), phosphate first and then borate/phosphate (●), tris/acetate (□), or tris/acetate first and then borate/phosphate (▲). The enzyme was assayed in the presence of 200 mM Pi (○, ●, □, ▲). All samples were inactive in the absence of Pi (◆).
product over time, while enzyme preincubated in the absence of phosphate (▲) showed a sigmoidal response, indicating that activation was occurring. Figure 25 shows a plot $\Delta P/t$ versus product formed (Boeker, 1982) for two of the curves from Figure 24*. Reactions that obey a hyperbolic rate law give a straight line result on this plot; this was observed for glutaminase preincubated with borate/phosphate (▲) but not in its absence (●). Thus, there was no time-dependent increase in borate/phosphate-incubated glutaminase activity, while activation of tris/acetate-incubated enzyme occurred during the assay.

Figure 26 shows the activation of glutaminase by increasing concentrations of phosphate. Depending on the initial state of the enzyme 60 to 180 mM phosphate was required to reach full activity.

In all of these experiments, dialysis of the enzyme against tris/acetate buffer and then against borate/phosphate buffer resulted in a recovery of only approximately 70% of the activity. The change which occurred in the absence of phosphate was apparently only partially reversible under these conditions.

If the activation of glutaminase by phosphate is due to a change in molecular weight, activation should depend on the protein concentration. This is shown in Figure 27. Various concentrations of glutaminase (0.1-0.3 mg/ml) $\Delta P = \triangleq \text{change in product with time}, P_t - P_0.$
Figure 25. Plot of $\Delta P/t$ versus product formed. Initial rate plot for two of the curves shown in Figure 24. Hyperbolic reactions give straight lines which intersect the ordinate at the initial rate of the reaction. The data shown resulted when glutaminase (1.2 mg/ml) was dialyzed against tris/acetate (●), or tris/acetate first and then borate/phosphate buffer (▲). The enzyme was assayed under otherwise standard assay conditions.
Figure 26. Activation of glutaminase as a function of the phosphate. Glutaminase (1 mg/ml) was dialyzed for 4 hours against borate/phosphate (●), tris/acetate (♦), or tris/acetate first and then borate/phosphate (▲) buffer. The enzyme was assayed for 90 minutes under standard conditions.
Figure 27. Effect of protein concentration on glutaminase activation. Glutaminase was dialyzed against borate/phosphate (●), or tris/acetate (▲, □) at different protein concentrations. The enzyme was assayed in the presence of 200 mM phosphate (●, ▲) for 20 minutes under otherwise standard assay conditions. Both samples were inactive in the absence of phosphate (□).
were dialyzed against borate/phosphate or tris/acetate buffer and were then assayed in the presence of 200 mM phosphate. A sigmoidal dependence of activity on protein concentration was observed for glutaminase preincubated in the absence of phosphate, whereas the dependence for glutaminase incubated with phosphate is normal.

Figure 28 shows that plots of activity against glutamine concentration are a function of pretreatment. The glutamine concentrations which gave half maximal activity for the results in Figure 28 were 30, 20, and 15 for the tris/acetate, phosphate, and borate/phosphate incubated enzymes, respectively. However, this experiment is in no way a formal measurement of $K_m$, as no attempt was made to measure initial rates.

Glutaminase which had been dialyzed in tris/acetate buffer showed a sigmoidal response to increasing glutamine concentrations during the assay as compared to the borate/phosphate or phosphate incubated enzyme. These results suggest that glutamine itself facilitates the activation by phosphate.

Effect of Different Ions on the Molecular Weight of Glutaminase

The molecular weight of cow brain glutaminase depends on the nature of the buffer against which it has been dialyzed. The enzyme molecular weight was determined in borate/phosphate, phosphate, or tris/acetate buffer as
Figure 28. Effect of glutamine concentration on glutaminase activity. Glutaminase (1.4 mg/ml) dialyzed against borate/phosphate (▲), phosphate (▲), or tris/acetate (▲,●) buffer. The enzyme was assayed for 120 minutes in the presence of 200 mM phosphate (▲,▲,▲). All samples were inactive in the absence of phosphate (▲).
shown in Figures 29-32. Calculations were carried out as suggested by Laurent & Killander (1964) by determining the fraction of the volume of the gel that is available for the substance ($K_{av}$) from the elution volume of the substance ($V_e$), the total volume of the gel bed ($V_t$) and the void volume ($V_0$). Various elution parameters such as $V_e$, $V_e/V_0$, and the distribution coefficient ($K_d$) have also been used in the literature for the preparation of calibration curves and molecular weight measurements. $K_{av}$ is preferred to other elution parameters since: (1) it is less sensitive to errors which may be introduced as a result of variations in column preparation and column dimensions and (2) it does not require the unreliable determination of the gel internal volume ($V_i$) as is required with $K_d$.

Figure 29 shows the gel filtration of glutaminase that was first equilibrated with borate/phosphate and then equilibrated and chromatographed with three buffers. In borate/phosphate, phosphate and tris/acetate, the enzyme eluted from Sepharose 4B at 13, 29 and 33% of the column volume respectively. If the enzyme was first equilibrated with tris/acetate, and then equilibrated and chromatographed in these three buffers, the enzyme in tris/acetate eluted from Sepharose 4B at 35% of the column volume (Figure 30), the enzyme in borate/phosphate elutes at 13% and that in phosphate at 31% of the column volume. Gel
Figure 29. Gel filtration of glutaminase on Sepharose 4B after preincubation in borate/phosphate. Panel A. After preincubation, glutaminase was dialyzed and chromatographed in borate/phosphate buffer. Panel B. Glutaminase was dialyzed and chromatographed in phosphate buffer. Panel C. Glutaminase was dialyzed and chromatographed in tris/acetate buffer. Additional details are given in Experimental Procedure.
Figure 30. Gel filtration of glutaminase on Sepharose 4B after preincubation in tris/acetate. Panel A. Chromatography in tris/acetate buffer. Panel B. Glutaminase from panel A dialyzed and chromatographed in phosphate buffer. Panel C. Glutaminase from panel A dialyzed and chromatographed in borate/phosphate buffers. Additional details are given in Experimental Procedure.
Figure 31. Gel filtration of glutaminase on Sepharose 6B after preincubation in borate/phosphate. Panel A. After preincubation, glutaminase was dialyzed and chromatographed in borate/phosphate buffer. Panel B. Glutaminase was dialyzed and chromatographed in phosphate buffer. Panel C. Glutaminase was dialyzed and chromatographed in tris/acetate buffer. Additional details are given in Experimental Procedure.
ACTIVITY, MOLES/MIN/ML ACTIVITY, MOLES/MIN/ML ACTIVITY, MOLES/MIN/ML

Absorbance 280 NM

Elution Volume, ML

ACTIVITY, MOLES/MIN/ML

Protein = ▼
Activity = ○

Tris/Acetate

Borate/Pi

Absorbance 280 NM

Absorbance 280 NM

Absorbance 280 NM
Figure 32. Calibration of the Sepharose 4B columns. Globular proteins of known molecular weights were used to calibrate the columns. The calibration points were determined in the tris/acetate (○), phosphate (●), and borate/phosphate (▲) buffers. Standards used were aldolase (158,000), catalase (232,000), ferritin (440,000), thyroglobulin (669,000). Kav was calculated according to equation: $K_{av} = (V_e - V_0)/(V_t - V_0)$, where $V_e$ is the elution volume of protein, $V_0$ is void volume of the column determined by running Blue Dextran through the column, and $V_t$ is the total bed volume.
filtration in these three buffers on Sepharose 6B increased the separation between the phosphate and tris/acetate forms, and decreased the separation between the borate/phosphate and phosphate forms (Figure 31). These results show that there were three forms of glutaminase, observed in tris/acetate, phosphate, and borate/phosphate buffers. These appeared to be interconvertible and have molecular weights of 170,000, 300,000, and >10^6 respectively (Table 4).

Figure 32 shows the calibration of Sepharose 4B columns which were used for molecular weight determinations. The observed $K_{av}$ values for the standard proteins were almost the same in borate/phosphate and phosphate buffers, but were different in the tris/acetate buffer. This is

Table 4. Summary of Glutaminase Molecular Weights.

<table>
<thead>
<tr>
<th>Column</th>
<th>Buffer</th>
<th>Elution volume at which activity eluted (ml)</th>
<th>Average Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sepharose 4B</td>
<td>Borate/Pi</td>
<td>13</td>
<td>&gt;1x10^6</td>
</tr>
<tr>
<td>Sepharose 4B</td>
<td>Borate/Pi</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Sepharose 4B</td>
<td>Phosphate</td>
<td>31</td>
<td>300,000</td>
</tr>
<tr>
<td>Sepharose 4B</td>
<td>Tris/Acetate + 100 mM Pi</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Sepharose 4B</td>
<td>Tris/acetate</td>
<td>33</td>
<td>170,000</td>
</tr>
<tr>
<td>Sepharose 4B</td>
<td>Tris/acetate</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>
probably due to the difference between the ionic strength of the buffers, since the borate/phosphate and phosphate buffers have ionic strengths greater than 1, while that of the tris/acetate buffer is only 0.01.
DISCUSSION

The procedure described for the purification of glutaminase from cow brain is reproducible and reasonably rapid. Because of the modified extraction conditions glutaminase has a greater initial specific activity than previously reported (Chiu, 1979). The final result is comparable both in milligrams of protein obtained and specific activity to that reported for pig brain glutaminase (Svenneby et al., 1973; Nimmo & Tipton, 1980); however, the overall yield of 22% is much greater.

Glutaminase stored in borate/phosphate buffer maintained a specific activity of approximately 142 μmoles/min/mg for at least six months. Upon dialysis against tris/acetate or phosphate buffer, the specific activity decreased four to six-fold and two-fold, respectively. Dialysis again against borate/phosphate buffer resulted in recovery of 70% or 100% of the initial activity. The degree of inactivation in tris/acetate buffer increased with the time of exposure and with decreasing protein concentration. Since inactivation also occurred in barbital buffer, it appears that phosphate exerted a protective effect. That is, inactivation was a result of the absence of phosphate, not the presence of tris/acetate or tris/Cl.

The molecular weight of cow brain glutaminase depends on the nature of the buffer in which it is stored. The molecular weight was found to be 170,000 in tris/acetate,
300,000 in phosphate and more than $10^6$ in phosphate/borate buffer. The subunit molecular weight estimated by SDS gel electrophoresis was 80,000. This suggests that glutaminase is a dimer in the absence of phosphate and a tetramer its presence. These results differ from those of Svenneby (1970) where pig brain glutaminase apparently does not undergo phosphate stimulated dimerization.

The results from polyacrylamide gel electrophoresis (Figure 21 A) along with the gel filtration data suggest that glutaminase in borate/phosphate buffer is highly aggregated. Although this form of glutaminase appears to give one protein band at the top of the stacking gel, there is no evidence that this band is homogeneous. It seems more likely, in fact, that it consists of a mixture of very high molecular weight polymers. Forms with molecular weight 170,000 or 300,000 run as two separate protein bands, one barely within the running gel and the other well down in the running gel. Changing the pH does not change the pattern. This suggests that the bands are due to molecular weight variations, not changes of charge. Since there is not a big difference in the molecular weights of the dimer and tetramer it seems unlikely that each band corresponds to one form. The protein band well down in the running gel could represent both the dimer and the tetramer, while the band between the stacking and running gel may correspond to polymers formed during electrophoresis, that
have somewhat lower molecular weights than the polymers in the upper band. It is possible that the two upper bands (polymers) have nonglobular structure or that they somehow interact with the polyacrylamide and do not enter the gel matrix.

The enzyme requires phosphate absolutely for activity. As shown in Figures 23, 24, and 26 the activity of the enzyme depends on Pi in the assay regardless of its presence or absence during preincubation.

Figures 24 and 25 show that when the dimer was assayed in the presence of phosphate, its initial rate of catalysis was zero. Preincubation of the dimer with phosphate resulted in a normal initial rate plot.

As shown in Figures 23, 24, and 26, addition of phosphate to the dimer caused a time dependent increase in the specific activity; phosphate also caused an increase in molecular weight, as shown in the gel filtration studies. As shown in Figure 27, the activity increase depended on the protein concentration. It is apparent that the dimer is inactive and that polymerization must precede or accompany activation.

In separate experiments, glutaminase in borate buffer was chromatographed on the Sepharose 4B column in the presence of this buffer. It appeared at the void volume. In addition, this enzyme was active when assayed in either phosphate or borate buffer but was inactive in tris or
The sigmoidal nature of the various kinetic curves is consistent with the fact that polymerization must occur before activity develops. Notice that in Figure 25, the activity requires some time to develop even in the presence of 0.2 M phosphate. This suggests that the sigmoidal
dependence of activity on phosphate (Figure 26) and glutamine (Figure 28) is due to appearance of activity during the assay itself rather than to a fundamentally sigmoid concentration dependence.

There may be two binding sites for phosphate one for polymerization and one for catalysis. Although it has proven difficult to separate polymerization and catalysis, the data suggest separate roles. If so phosphate would be acting as an allosteric activator during catalysis.

A number of kinetic experiments in addition to those shown here suggest that at least two binding sites each for phosphate and borate are involved (Svenneby, 1971; Chiu & Boeker, 1979; Kvamme & Torgner, 1974; Nimmo & Tipton, 1981). This has never been tested directly. Therefore one could perform equilibrium dialysis and/or titration calorimetry experiments to determine how many phosphates or borates can be bound per subunit and whether or not sites with more than one binding constant are involved. Competition experiments between the various effectors should then indicate whether any of the sites are specific for particular compounds.
LITERATURE CITED


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- Standard procedures for standardization foods and drinks 245 pages
- Organic Chemistry 183 pages
- General Biochemistry 300 pages
- Experimental Biochemistry 109 pages