Interaction of Substrates with Glutamine Synthetase after Limited Proteolysis†

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ABSTRACT: Previous studies [Dautry-Varsat, A., Cohen, G. N., & Stadtman, E. R. (1979) *J. Biol. Chem. 254*, 3124–3128; Lei, M., Aebi, U., Heidner, E. G., & Eisenberg, D. (1979) *J. Biol. Chem. 254*, 3129–3134] have shown that *Escherichia coli* glutamine synthetase (GS) can be cleaved by proteases to form a limited digestion species called nicked glutamine synthetase (GS*). The present study gives the amino acid sequence of the protease-sensitive region of glutamine synthetase. The present study also shows that GS* is enzymatically active, but this activity is low compared to the activity of GS. The apparent Michaelis constant value for glutamate was 90 mM for GS* as compared to 3 mM for GS, while the Michaelis constant values for ATP were similar for GS and GS*. The dissociation constant values for ATP, as determined by intrinsic fluorescence measurements, were similar for GS and GS*. Glutamate decreased the dissociation constant value of ATP for GS because of synergism between the two binding sites; glutamate did not decrease the dissociation constant value of ATP for GS*. The glutamate analogue methionine sulfoximine bound very tightly to GS and inactivated the enzyme in the presence of ATP. Methionine sulfoximine did not appear to bind to GS* and did not inactivate GS* in the presence of ATP. The ATP analogue 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine bound to GS and inactivated the enzyme by forming a covalent bond with it. Glutamate accelerated this inactivation because of the synergism between the ATP and glutamate binding sites of GS. 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine bound to GS* at least as tightly as to GS, but inactivated GS* 10-fold more slowly than GS. Glutamate did not increase the rate of inactivation of GS* by 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine. The present study has shown that the lower enzymatic activity of GS* compared to GS is due to an apparent decrease in the binding affinity of GS* for glutamate and to a loss of the synergism between ATP and glutamate binding.

Glutamine synthetase (GS)[1] [L-glutamate:ammonia ligase (ADP forming); EC 6.3.1.2] is the central enzyme in nitrogen regulation in bacteria. The enzyme fixes ammonia to glutamate to form glutamine with ATP being cleaved in the process. GS is subject to a number of control mechanisms including allosteric effectors, metal ion specificity, and adenylylation (Ginsburg, 1972; Stadtman, 1971; Stadtman & Ginsburg, 1974). The enzyme in its active form exists as a dodecamer of identical subunits (Kingdon et al., 1972), each having a molecular weight of 50000. The subunits form two hexameric rings stacked face to face (Valentine et al., 1968). For a review of the properties of glutamine synthetase, see Ginsburg (1972) and Stadtman & Ginsburg (1974).

Glutamine synthetase has an intrinsic fluorescence due to the presence of two to three tryptophanyl residues (Timmons et al., 1974). The intrinsic fluorescence of the enzyme changes on addition of metal ion, substrates, and some substrate analogues. Fluorescence changes in the presence of the glutamate analogue methionine sulfoximine (Weisbrod & Meister, 1973) have shown intersubunit cooperativity (Rhee et al., 1981).

Glutamine synthetase is susceptible to limited proteolysis, termed nicking, under nondenaturing conditions by a variety of proteases (Dautry-Varsat et al., 1979; Lei et al., 1979). Proteolytic nicking occurs within a limited region of the enzyme and leads to a 97% reduction of enzymatic activity as measured by transferase and forward assays. Nicked glutamine synthetase (GS*) retains its overall dodecameric structure, is still recognized by antibodies to intact enzyme, and is still adenylylated essentially normally (Dautry-Varsat et al., 1979; Lei et al., 1979). GS* differs from other enzymatically active nicked enzymes in that most of these are multifunction enzymes that are separated into two or more functional domains, each having some portion of the original activity [for a review, see Kirschner & Bisswanger (1976) or Kirschner & Szadkowski (1980)]. GS* shows similarities to the β chain of tryptophan synthetase, which can be nicked with trypsin to give a pair of interacting globular domains that, though inactive, can still bind coenzyme and substrates (Högberg-Raibaud & Goldberg, 1977).

While GS* appears to retain a number of the properties of the intact enzyme, preliminary studies indicated that GS* shows at least one major difference in that it is not inactivated by methionine sulfoximine in the presence of ATP (Monroe et al., 1983). Methionine sulfoximine (a glutamate analogue) and ATP interact at the active site of intact GS to form a methionine sulfoximine phosphate–ADP complex that associates with GS so tightly that the complex inactivates the subunit it is bound to (Rowe et al., 1969). Because of intersubunit cooperativity, the dodecamer is inactivated when only 9–11 subunits have methionine sulfoxime bound (Rhee et al., 1981).

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[1] Abbreviations: GS, glutamine synthetase; GS*, protease-nicked glutamine synthetase; FSO_2BzAdo, 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine; Met(O)(NH), methionine sulfoximine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; M, metal.

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The ATP analogue 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (Pal et al., 1975) has been used in our laboratory to examine the ATP binding site of GS (Foster et al., 1981). One mole of 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine per mole of subunit is covalently attached to the enzyme at a site near the site of interaction of the γ-phosphate of ATP. The dissociation constant of 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine with GS is nearly equal to the dissociation constant of ATP with the enzyme. The rate of inactivation of glutamine synthetase by 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine is enhanced by glutamate due to an intrasubunit interaction referred to as synergism of binding (Rhee & Chock, 1976). This synergism of binding can also be seen in the binding of the substrates glutamate and ATP (Timmons et al., 1974).

The purpose of the present study was to determine why nicked glutamine synthetase showed lower activity than intact glutamine synthetase, and to determine the amino acid sequence at the nicking site. The dissociation constants of ATP and glutamate for GS and GS* were determined by activity assay and by intrinsic fluorescence studies. The ATP and glutamate binding sites were also examined by using the ATP analogue 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine and the glutamate analogue methionine sulfoximine.

Materials and Methods

Materials. *E. coli* used in the present studies was the gracious gift of Dr. David Novelli, E. F. Phares, and Mary Long of the Oak Ridge National Laboratory and was grown by the procedure of Phares (1971). Electrophoresis chemicals, including acrylamide and sodium dodecyl sulfate, were of electrophoresis grade and were purchased from Bio-Rad. ATP, ADP, glutamate, glutamine, imidazole, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma. All organic solvents were purchased from Fisher. 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine (FSO_2BzAdo) and L-methionine sulfoximine [Met(O)(NH)] were purchased from Sigma and were used without further purification. The concentration of 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine in dimethyl sulfoxide was determined spectrophotometrically by measuring the absorbance at 232 ($\epsilon = 18.8\ mM^{-1}\cdot cm^{-1}$) and 259 nm ($\epsilon = 13.5\ mM^{-1}\cdot cm^{-1}$) as described by Wyatt & Coleman (1977). Cibacron Blue–agarose was prepared as described previously (Travis et al., 1976). *Staphylococcus aureus* V8 was purchased from Miles. All other proteases were purchased from Worthington. All other chemicals were reagent grade.

Isolation of Glutamine Synthetase. Glutamine synthetase was purified from *E. coli* by a modification of the procedure of Woolfolk et al. (1966) where the heat treatment step was replaced by affinity chromatography over Cibacron Blue–agarose. This procedure is similar to the purification procedure described by Burton et al. (1981). GS was determined to be homogeneous by SDS–acrylamide gel electrophoresis and by amino-terminal amino acid sequence analysis.

The concentration of GS was determined spectrophotometrically by using a Beckman Acta CIII spectrophotometer and extinction coefficient values of $\epsilon_{280} = 0.737\ mL\cdot mg^{-1}\cdot cm^{-1}$ and $\epsilon_{290} = 0.383\ mL\cdot mg^{-1}\cdot cm^{-1}$ (Hunt et al., 1972). The state of adenylylation was determined spectrophotometrically by the ratio of the absorbance values at 260 and 290 nm (Shapiro & Stadtman, 1970). Absorbance values were corrected for light scatter (Hunt et al., 1972). The state of adenylylation was also determined by assay as described by Stadtman et al. (1979). Both procedures gave a state of adenylylation of 1.7.

The concentration of nicked glutamine synthetase was determined spectrophotometrically as above, assuming the extinction coefficients were the same as those for GS. For GS, the molecular weight was assumed to be 600 000 for the dodecamer and 50 000 for the monomer subunit (Valentine et al., 1968). For GS*, a subunit was considered to be an associated pair of 31 000 and 15 000 molecular weight fragments (see Results). Calculations of the number of active sites in GS* determined from ATP binding by intrinsic fluorescence studies (described below) correlated well with the number of sites expected from spectrophotometric measurements, assuming one active site per associated 31 000 and 15 000 molecular weight fragment.

Preparation of Nicked Glutamine Synthetase. GS, 1–4 mg/mL (0.02–0.08 mM in subunits), in a solution of 10 mM imidazole and 10 mM $MnCl_2$ at pH 7 was incubated with 1% (w/w) trypsin, chymotrypsin, or *Staphylococcus aureus* V8 protease at room temperature. At various times, 0.01-mL samples were removed and residual enzymatic activity was determined by using either the transferase or the forward assay (described below). Enzyme activity appeared constant at about 1% of the initial activity after 2 h. GS* was separated from the protease by gel filtration using either a Spherogel-TSK 3000SW high-pressure liquid chromatography column or a Sephadex G-75 column equilibrated with 10 mM HEPES, 25 mM $MgCl_2$, and 100 mM KCl. GS* was stored as a suspension in 50% ammonium sulfate.

In some cases, GS* was treated with 10 mM methionine sulfoximine and 10 mM ATP for 1 h to inactivate residual intact glutamine synthetase. In these cases, trypsin was inactivated by adding diisopropyl fluorophosphate (1 mM) to the solution (Balls & Jansen, 1952) before adding the methionine sulfoximine and ATP. The GS* was gel filtered through the columns described above to remove the substrates and diisopropyl fluorophosphate. The diisopropyl fluorophosphate had no effect on the GS* activity.

Amino-Terminal Sequence Analysis. Automated Edman degradation was performed by using a Beckman 890C sequencer with a 0.1 M Quadrol program (Brauer et al., 1975). Phenylthiohydantoin-amino acids were identified by reverse-phase high-performance liquid chromatography on an Ultrasphere–ODS column (0.46 × 25 cm) in a sodium acetate, pH 4.9, and acetonitrile solvent gradient (Noyes, 1983).

Enzyme Activity Assay. Both GS and GS* will catalyze the following reactions that are dependent on the presence of divalent cations.

$$\text{glutamate} + \text{ATP} + NH_3 \xrightarrow{M^{2+}} \text{glutamine} + \text{ADP} + P \quad (1)$$

$$\text{glutamine} + NH_2OH \xrightarrow[M^{2+}]{\text{ADP, } AsO_4} \gamma\text{-glutamylhydroxamate} + NH_3 \quad (2)$$

The conditions for assaying the activity of GS by reaction 1 (forward assay) were 50 mM imidazole, 7.5 mM ATP, 100 mM glutamate, 50 mM NH_4Cl, and 25 mM $MgCl_2$ (Woolfolk et al., 1966). The reaction was stopped by addition of 1.8 mL of a freshly prepared solution of 0.8 g of $FeSO_4\cdot 7H_2O$ in 100 mL of 0.015 N H_2SO_4. Color was developed for 1 min by addition of 0.15 mL of 6.6% $(NH_2)_6Mo_7O_{24}\cdot 7H_2O$ in 7.5 M H_2SO_4. The molybdate phosphate complex that is formed has an absorbance maximum at 660 nm. To assay GS*, the ATP concentration was increased to 15 mM and the glutamate concentration to 200 mM.

The conditions for assaying the activity of GS by reaction 2 (transferase assay) were 50 mM triethanolamine–dimethylglutarate, 150 mM glutamine, 20 mM NH_2OH (freshly neutralized with KOH before addition), 20 mM KCl, 0.2 mM

ADP, and 20 mM K_2AsO_4 (Stadtman et al., 1979). The mixture was adjusted to pH 7.57 with triethanolamine, and then $MnCl_2$ was added to a final concentration of 0.4 mM. Enzyme (0.02 mL) was added to 0.48 mL of the above solution. The reaction was stopped by adding 2 mL of a solution of 8% trichloroacetic acid, 2 N HCl, and 3.3% $FCl_3 \cdot H_2O$. The $FeCl_3$ reacts with γ-glutamylhydroxamate to give an iron hydroxamate complex that has an absorbance maximum at 540 nm. At pH 7.57, in the presence of Mn^{2+}, adenylylated and unadenylylated subunits of GS are equally active. All conditions were the same for assays on the activity of GS*. For assays of the activity of GS and GS* as a function of pH, $MnCl_2$ and $MgCl_2$ were added to the assay solution after the pH had been adjusted. The addition of 0.4 mM Mn^{2+} did not change the pH of the assay solution, but addition of 60 mM Mg^{2+} lowered the pH of the assay solution 0.2 unit (Stadtman et al., 1979). The assay solutions containing Mn^{2+} measured the activity of both adenylylated and unadenylylated subunits while the assay solutions containing Mn^{2+} plus Mg^{2+} measured the activity of the unadenylylated subunits only.

Since GS and GS* catalyze the same reactions, but at different rates, an incomplete nicking of GS to GS* will result in a mixture of two enzyme species that have different kinetic parameters for the same reaction. When an enzymatic reaction is catalyzed by two enzyme species, each having a different K_m and V_{max}, a plot of substrate concentration divided by reaction velocity vs. substrate concentration will be nonlinear (Segel, 1975).

$$v = \frac{V_{max}[S]}{K_m + [S]} + \frac{V_{max}'[S]}{K_m' + [S]} \quad (3)$$

It is possible, using a regression analysis, to separate the activity of these two species and define the K_m and V_{max} for each (Spears et al., 1971).

Reactions with Methionine Sulfoximine. The interaction of GS and GS* with methionine sulfoximine was followed by the loss of activity in the forward and transferase assays (described above). GS (0.01 μM) or GS* (1 μM) was incubated in 10 mM methionine sulfoximine, 10 mM ATP, 25 mM HEPES, pH 7.2, and 10 mM $MgCl_2$. At various times, samples were removed and assayed for enzymatic activity. GS* was separated from methionine sulfoximine and ATP by gel filtration on a Sephadex G-50 column in 25 mM HEPES–10 mM $MgCl_2$.

Intrinsic Fluorescence Changes on Substrate Binding. Intrinsic fluorescence measurements on native and nicked glutamine synthetase were made by using a Turner Model 430 spectrofluorometer equipped with a wavelength drive and attached to a Fisher Recordall series 5000 recorder. The protein concentration was 0.6 mg/mL (0.012 mM) in a solution containing 25 mM HEPES, pH 7.2, 25 mM $MgCl_2$, 100 mM KCl, and substrates. All experiments were performed at 20 °C with an excitation wavelength of 300 nm. The emission was scanned from 450 to 310 nm. At an excitation wavelength of 300 nm, there should be no fluorescence due to ATP. Binding constants for ATP under all conditions were determined by making 0.002-mL additions of stock solutions of ATP; since the total volume of ATP added never exceeded 1% of the total enzyme solution volume, no correlation for dilution was made.

Reactions with 5′-[p-(Fluorosulfonyl)benzoyl]adenosine. The reaction of GS* with 5′-[p-(fluorosulfonyl)benzoyl]-adenosine was followed by the loss of forward reaction activity with time. The GS* used in this study had been treated with methionine sulfoximine as described above. If untreated GS* was used, the log $([E]/[E]_0)$ vs. time plots were curved because the two enzymes species present, GS and GS*, lost activity at different rates. GS* in 25 mM HEPES, 25 mM $MgCl_2$, 100 mM KCl, 5% (v/v) ethanol, and 5% (v/v) dimethyl sulfoxide was incubated at 4 °C with various concentrations of 5′-[p-(fluorosulfonyl)benzoyl]adenosine. The reaction solutions contained ethanol and dimethyl sulfoxide to ensure the solubility of the 5′-[p-(fluorosulfonyl)benzoyl]adenosine (Foster et al., 1981). Even with the organic solvents present, 5′-[p-(fluorosulfonyl)benzoyl]adenosine was not soluble above 0.5 mM (Foster et al., 1981). At various times, 0.02-mL samples were removed from the reaction mixture, and residual activity was determined in a forward assay as described for GS*. The large excess of ATP was adequate to compete with the 5′-[p-(fluorosulfony)benzoyl]adenosine and prevent further inactivation.

For studies on the competition of 5′-[p-(fluorosulfonyl)-benzoyl]adenosine with ATP in the forward assay, assay solutions contained 5% (v/v) ethanol, 5% (v/v) dimethyl sulfoxide, 100 mM NH_4Cl, 200 mM glutamate, 50 mM imidazole, and 50 mM $MgCl_2$ with varying concentrations of ATP and 5′-[p-(fluorosulfonyl)benzoyl]adenosine.

Determination of the Kinetic Parameters for the Inactivation of GS by 5′-[p-(Fluorosulfonyl)benzoyl]adenosine.* GS* was added to a solution containing 10 mM imidazole (pH 7.0), 10 mM $MgCl_2$, 100 mM KCl, 5% ethanol, 5% dimethyl sulfoxide, and 5′-[p-(fluorosulfonyl)benzoyl]adenosine. The final GS* concentration was 0.5 mg/mL (10 μM in subunits), and the 5′-[p-(fluorosulfonyl)benzoyl]adenosine concentration was varied. At time intervals, 0.02-mL samples were removed and added to a solution containing 50 mM imidazole (pH 7.0), 7.5 mM ATP, 100 mM glutamate, 50 mM NH_4Cl, and 25 mM $MgCl_2$, and the rate of phosphate formation was determined as described above (see Enzyme Activity Assay). Previous work in our laboratory (Foster et al., 1981) has shown that the 5′-[p-(fluorosulfonyl)benzoyl]adenosine reaction with native GS follows pseudo-first-order kinetics as described by the following equation:

$$\ln \frac{[GS]}{[GS]_0} = -k'_{obsd}t \quad (4)$$

where $[GS]_0$ is the initial native GS concentration, [GS] is the concentration of native GS at reaction time t, and k'_{obsd} is the apparent pseudo-first-order rate constant observed at a given 5′-[p-(fluorosulfonyl)benzoyl]adenosine concentration. The 5′-[p-(fluorosulfonyl)benzoyl]adenosine–GS reaction is saturable with respect to 5′-[p-(fluorosulfonyl)benzoyl]-adenosine, which is consistent with the mechanism described by the following equation:

$$GS + FSO_2BzAdo \overset{K_I}{\rightleftharpoons} GS \cdot FSO_2BzAdo \xrightarrow{k_2} GS\text{–}FSO_2BzAdo$$

where K_I is the apparent dissociation constant for the noncovalent GS·5′-[p-(fluorosulfonyl)benzoyl]adenosine complex and k_2 is the first-order rate constant for the formation of the covalent GS–5′-[p-(fluorosulfonyl)benzoyl]adenosine complex. The rate equation for the reaction is described by the following equation:

$$k_{obsd} = \frac{k_2[FSO_2BzAdo]}{K_I + [FSO_2BzAdo]} \quad (5)$$

which can be rearranged to

$$\frac{1}{k_{obsd}} = \frac{K_I}{k_2} \frac{1}{[FSO_2BzAdo]} + \frac{1}{k_2} \quad (6)$$

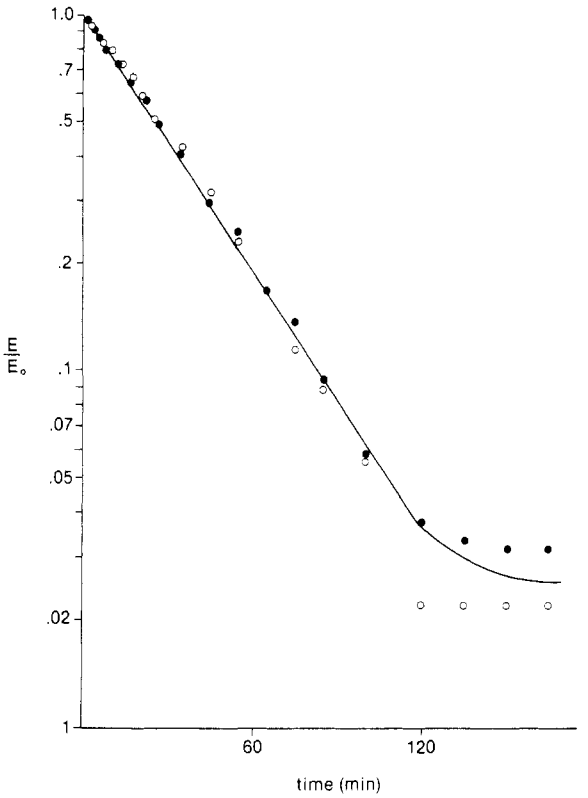

FIGURE 1: GS activity as a function of incubation time with trypsin. Glutamine synthetase was incubated with 1% (w/w) trypsin in 10 mM imidazole, pH 7.5, and 10 mM $MgCl_2$ at 22 °C. At the times shown, samples were removed and assayed for activity (as described under Materials and Methods) in either the forward assay (●) or the transferase assay (○).

5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine is also a competitive inhibitor of the forward reaction catalyzed by native GS, with respect to ATP (Foster et al., 1981). To evaluate the effect of 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine on the kinetics of the forward reaction catalyzed by GS*, GS* was added to a solution containing 50 mM imidazole (pH 7.2), 100 mM glutamate, 50 mM NH_4Cl, 25 mM $MgCl_2$, ATP, and 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine, and the rate of phosphate formation was determined as described above. The GS* concentration was 0.5 mg/mL (10 μM in subunits), and the ATP and 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine concentrations were varied. The rate equation for the forward reaction, assuming competitive inhibition by 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine with respect to ATP, is described by the following equation:

$$v_i = \frac{V_{max}[ATP]}{K_m(1 + [FSO_2BzAdo]/K_i) + [ATP]} \quad (7)$$

which can be rearranged to

$$\frac{[ATP]}{v_i} = \frac{1}{V_{max}}[ATP] + \frac{K_m(1 + [FSO_2BzAdo]/K_i)}{V_{max}} \quad (8)$$

where K_i is the apparent dissociation constant for 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine and V_{max} is the maximum reaction velocity at saturation with respect to ATP. From eq 7, the apparent K_m value for ATP is described by the following equation:

$$K_{m,app} = \frac{K_m}{K_i}[FSO_2BzAdo] + K_m \quad (9)$$

Table I: Sequence of the Protease-Sensitive Region of GS

$_{a}$Glu^{1}-$_{b}$Gly-Gly-Asn-Lys^{5}-$_{c}$Gly-His-Arg-Pro-Ala^{10}-
Val-Lys-$_{c}$Gly-Gly-Tyr^{15}-$_{a}$Phe-Pro-Val-Pro-Pro^{20}-
Val-Asp-Ser-Ala-Gln^{25}-Asp-Ile-Arg-Ser-
Glu^{30}-Met-Ser-Leu-Val-Met^{35}-Glu-Gln-Met-

[a] Chymotrypsin cleavage point. [b] *Staphylococcus aureus* protease cleavage point. [c] Trypsin cleavage point.

Results

Characteristics of the Proteolytic Cleavage Sites on Glutamine Synthetase. When glutamine synthetase was incubated with trypsin, chymotrypsin, or *Staphylococcus aureus* protease (Dautry-Varsat et al., 1979; Lei et al., 1979) as described under Materials and Methods, the enzymatic activity, as measured by both transferase and forward assays, decreased in a pseudo-first-order pattern until only 1–3% of the initial activity remained (Figure 1). SDS–polyacrylamide gel electrophoresis (Laemmli, 1970; LeStourgen & Beyer, 1977) of samples taken at various times during the incubation indicated that the 50 000 molecular weight subunit was cleaved into two smaller fragments of apparent molecular weights 31 000 and 15 000. The total molecular weight of the two fragments is not 50 000 because a small peptide is released during nicking (Lei et al., 1979).

The 31 000 and 15 000 molecular weight fragments were separated on a Sephadex G-75 column in 0.1% formic acid. Under these conditions, the 15 000 molecular weight fragment eluted before the 31 000 molecular weight fragment, as demonstrated by polyacrylamide gels; the larger fragment eluted somewhat later than 1 column volume. Amino-terminal amino acid sequence analysis showed that the amino-terminal sequence of the 15 000 molecular weight fragment corresponded to that of intact glutamine synthetase. The 31 000 molecular weight fragment from incubation of GS with trypsin had an amino-terminal sequence of Gly-Gly-Tyr-Phe-Pro-Val-Pro-Pro-. The 31 000 molecular weight fragment from incubation of GS with chymotrypsin had an amino terminal of Phe-Pro-Val-Pro-Pro- (identical with the sequence starting at residue 4 of the trypsin sequence). The 31 000 molecular weight fragment from incubation of GS with *Staphylococcus aureus* protease had an amino-terminal sequence of Gly-Gly-Asn-Lys-Gly-.

When the mixture containing nicked glutamine synthetase and trypsin was run through a Sephadex G-75 column (2.6 cm × 30 cm) in HEPES buffer at pH 7, a small peptide was isolated. The complete sequence of this peptide was Gly-His-Arg-Pro-Ala-Val-Lys (residues 5–11 of the amino-terminal sequence from the 31 000 molecular weight fragment generated by *Staphylococcus aureus* protease digestion). The sequence data are summarized in Table I.

Effect of pH on GS Activity.* GS and GS* activity in the transferase assay was determined as a function of pH (Figure 2). In the presence of Mn^{2+}, the pH optimum was 8.2 for GS and 7.05 for GS*. In the presence of Mn^{2+} plus Mg^{2+}, the pH optimum was 7.8 for GS and 6.7 for GS*.

Enzymatic Activity of Nicked Glutamine Synthetase. The kinetic parameters for the forward reaction catalyzed by GS and GS* were determined by measuring the initial reaction rate as a function of glutamate (GLU) concentration. With GS, the apparent K_m^{GLU} and k_{cat} values were 3 mM and 830 mol of phosphate formed min^{-1} (mol of GS)$^{-1}$, respectively, when the glutamate concentration was varied in the presence of 10 mM ATP (Figure 3). Also shown in Figure 3 is the plot of the data obtained with GS*. The plot is nonlinear, suggesting the presence of two (or more) enzyme species. This

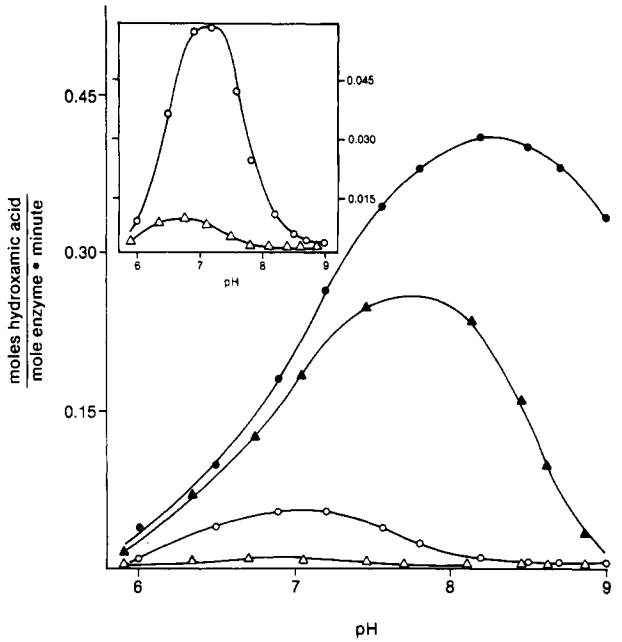

FIGURE 2: Effect of pH on the transferase activity of GS and GS*. GS or GS* was incubated in a transferase assay solution (described under Materials and Methods) at the pH values indicated. In the triethanolamine–dimethylglutarate buffer used for the transferase assay, only unadenylylated subunits are active in the presence of Mn^{2+} plus Mg^{2+}, while both adenylylated and unadenylylated subunits are active in the presence of only Mn^{2+} (Stadtman et al., 1979). Shown is the activity of GS in the presence of 0.4 mM Mn^{2+} (●, both subunits) or 0.4 mM Mn^{2+} plus 60 mM Mg^{2+} (▲, unadenylylated subunits) and the activity of GS* in the presence of 0.4 mM Mn^{2+} (○, both subunits) or 0.4 mM Mn^{2+} plus 60 mM Mg^{2+} (△, unadenylylated subunits). The inset shows the activity of GS* on an expanded scale.

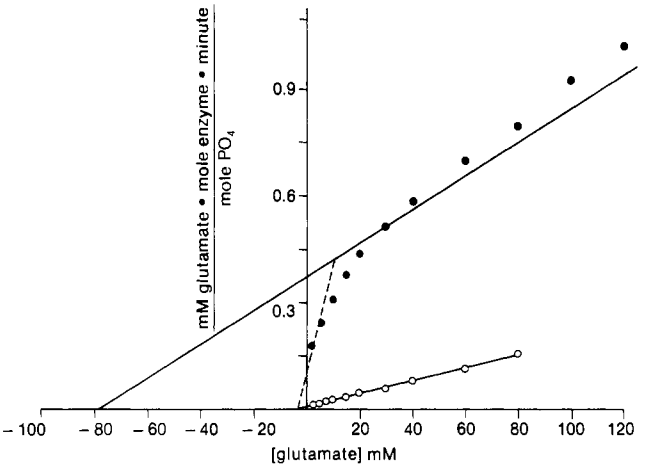

FIGURE 3: Dependence of the activity of GS and GS* on the concentration of glutamate. GS* was assayed for activity in a forward assay solution (described under Materials and Methods) containing the indicated concentration of glutamate (●). The data were plotted according to the equation $[S]/v = (1/V_{max})[S] + K_m/V_{max}$. The fitted lines were derived as described under Materials and Methods. GS was also assayed for activity in the forward assay (○).

result would be expected if the GS* preparation contained a trace amount of native GS. The data obtained with GS* were therefore subjected to a regression analysis to distinguish the kinetic parameters for GS* from those for GS (see Materials and Methods). With this approach, the apparent K_m^{GLU} value was calculated to be 80 mM for one enzyme species (solid line in Figure 3). The apparent K_m^{GLU} for the second enzyme species (native GS) was 3 mM (dashed line in Figure 3). From these data and from the known k_{cat} values, it appeared that

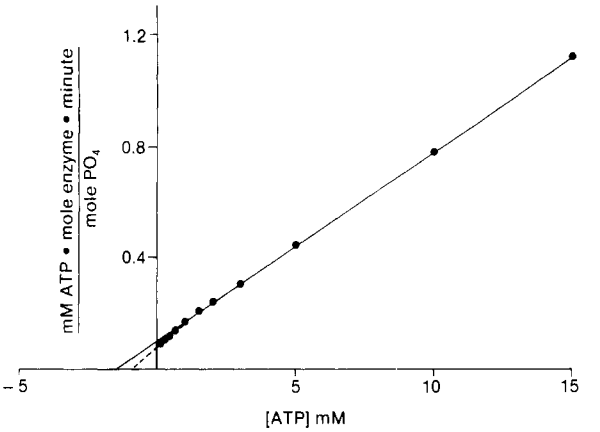

FIGURE 4: Dependence of the activity of GS* on the ATP concentration. GS* were assayed for activity in a forward assay solution (described under Materials and Methods) containing the indicated concentrations of ATP. The data were plotted according to the equation $[S]/v = (1/V_{max})[S] + K_m/V_{max}$. The fitted lines were derived as described under Materials and Methods.

the GS* preparation was contaminated by the presence of 0.1% (w/w) native GS.

The kinetic parameters for the forward reaction catalyzed by GS and GS* were also determined by measuring the initial reaction rate as a function of ATP concentration and plotting the data as shown in Figure 4. The plot of the data obtained with GS* was only slightly nonlinear, suggesting that the apparent K_m^{ATP} values for GS and GS* are very similar, 1.3 mM.

*Reaction of Methionine Sulfoximine with GS**. The intrinsic fluorescence of GS* did not increase when methionine sulfoximine was added. Even in the presence of ATP, methionine sulfoximine did not increase the intrinsic fluorescence of GS*. These results are unlike those seen with GS where methionine sulfoximine alone caused a 65% increase in intrinsic fluorescence and with ATP gave a 30% increase in intrinsic fluorescence over that due to ATP alone. GS*, incubated with ATP and methionine sulfoximine as described under Materials and Methods, showed an initial rapid loss of only 10% of its activity, after which the remaining activity was constant for at least 24 h.

The kinetic parameters for the forward reaction catalyzed by methionine sulfoximine·ATP-treated GS* were determined as described above. The apparent K_m^{GLU} and K_m^{ATP} values were 90 and 1.3 mM, respectively, with no evidence for the nonlinearity in the plots as was observed in Figures 3 and 4. The K_m^{GLU} value calculated from regression analysis (80 mM, Figure 3) agrees well with the K_m^{GLU} value determined with methionine sulfoximine·ATP-treated GS*.

Intrinsic Fluorescence of GS. GS shows an intrinsic fluorescence due to two to three tryptophanyl residues per subunit (Timmons et al., 1974). GS* also shows an intrinsic fluorescence, and when the concentrations of GS and GS* were the same, the relative fluorescence intensities of GS and GS* were the same. The addition of ATP caused a 50% increase in the relative fluorescence. Subsequent addition of glutamate caused a second 25% increase in fluorescence. Addition of ammonia to this GS–ATP–glutamate complex caused a loss of fluorescence to a level slightly below that of the GS plus ATP (Timmons et al., 1974) (Figure 5A). When ATP was added to GS*, there was a relative fluorescence increase of only 35%. Glutamate added to this GS*–ATP complex gave no further increase in fluorescence. The subsequent addition of ammonia to this GS*–ATP–glutamate gave a 5% decrease in fluorescence. ATP, glutamate, and ammonia constitute a

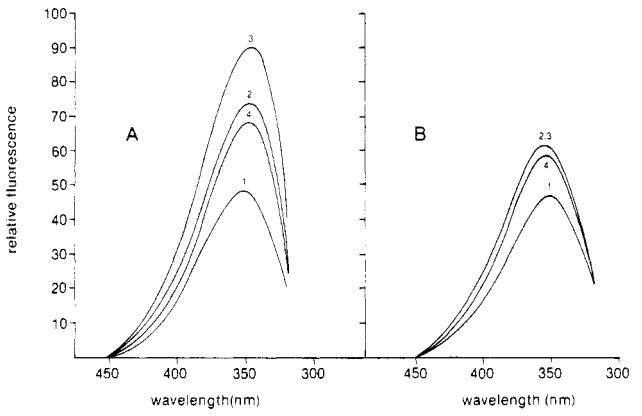

FIGURE 5: Intrinsic fluorescence changes in GS and GS* on addition of substrates. The intrinsic fluorescence of GS (A) and GS* (B) was determined (as described under Materials and Methods) in the absence of substrates (1) and in the presence of the substrates 10 mM ATP (2), 10 mM ATP and 200 mM glutamate (3), and 10 mM ATP, 200 mM glutamate, and 50 mM NH_4 (4).

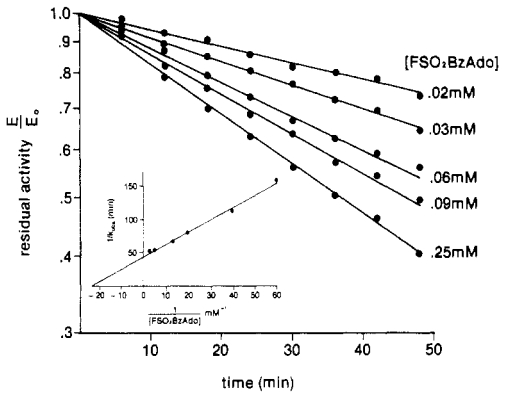

FIGURE 6: Inactivation of GS* by 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine. GS* was incubated with the indicated concentration of 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine in 10 mM imidazole, pH 7.0, 10 mM $MgCl_2$, 100 mM KCl, 5% ethanol, and 5% dimethyl sulfoxide as described under Materials and Methods. At the times shown, samples were removed and assayed for residual forward activity as described under Materials and Methods. The data were plotted according to the equation $\log([E]/[E]_0) = (-k_{obsd}/2.303)t_0$. The inset shows the k_{obsd} values plotted according to the equation $1/k_{obsd} = (K_I/k_2)(1/[I]) + 1/k_2$.

complete reaction mix that the enzyme catalyzes to form ADP, phosphate, and glutamine, so the complex of the complete mix gives a level of fluorescence identical with the level obtained by adding ADP plus phosphate to GS* (Figure 5B). The addition of glutamate alone to GS gave essentially no change in fluorescence; ATP added to this GS–glutamate complex gave an increase in the relative fluorescence to the level achieved by first adding ATP and then glutamate. Glutamate alone added to GS* gave no increase in the fluorescence; the subsequent addition of ATP gave a fluorescence increase to the level of ATP alone.

The value of the dissociation constant of ATP for GS was 0.1 mM as determined by titrating the increase in fluorescence on addition of ATP. The dissociation constant of ATP for the GS–glutamate complex is lower by a factor of 6 (0.02 mM) due to a synergism of binding between ATP and glutamate (Timmons et al., 1974). With GS*, the dissociation constant for ATP was 0.14 mM. The dissociation constant for ATP in the presence of glutamate was essentially unchanged (0.18 mM).

*Reaction of 5′-[p-(Fluorosulfonyl)benzoyl]adenosine with GS**. When GS* was reacted with 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine as described under Materials and Methods,

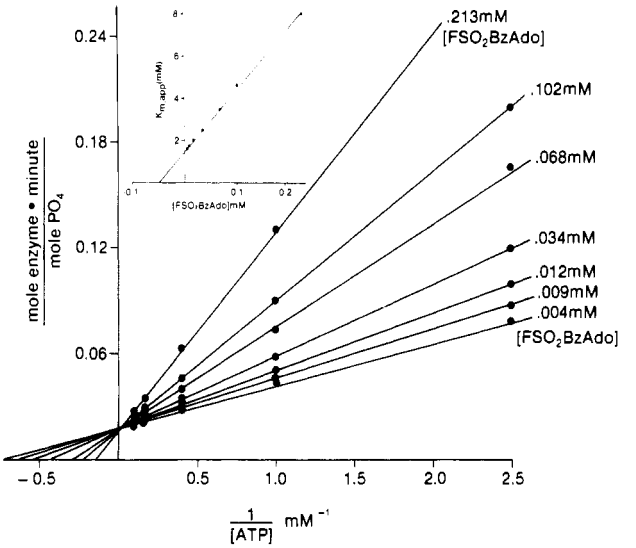

FIGURE 7: Competitive inhibition of GS* by 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine with respect to ATP. GS* was added to a solution containing 50 mM imidazole, pH 7.0, 200 mM glutamate, 50 mM NH_4Cl, 25 mM $MgCl_2$, ATP, and 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine at 22 °C. The ATP and 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine concentrations were varied as indicated. The rate of phosphate formation was determined as described under Materials and Methods. The data were plotted according to the equation $1/v = (K_{m,app}/V_{max})(1/[S]) + 1/V_{max}$. The inset shows the determined $K_{m,app}$ values plotted according to the equation $K_{m,app} = (K_m/K_I)[I] + K_m$.

the loss of activity showed pseudo-first-order kinetics as shown in Figure 6. The GS*–5′-[*p*-(fluorosulfonyl)benzoyl]adenosine reaction was saturable with respect to 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine with apparent K_1 and k_2 values of 0.05 mM and 0.023 min^{-1}, respectively. Similar experiments with native GS indicated apparent K_I and k_2 values of 0.22 mM and 0.23 min^{-1}, respectively. 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine was also a competitive inhibitor of the forward reaction with respect to ATP. The apparent K_i value for 5′-[*p*-(fluoro-sulfonyl)benzoyl]adenosine·GS* was 0.05 mM as determined from the data shown in Figure 7.

Discussion

Proteolysis of glutamine synthetase under nondenaturing conditions by trypsin, chymotrypsin, or *Staphylococcus aureus* protease occurs in a limited region (Table I). No digestion occurs beyond the Pro-Val-Pro-Pro portion of the large fragment with any of the proteases used. That combination of three prolines in four residues probably "kinks" the peptide chain and may serve to bury the peptide chain at the end of the exposed region of sequence while holding the susceptible region in an exposed configuration. Also, the exposed sequence is flanked by a pair of glycine residues (residues 2–3 and 13–14). This amino acid is the least hindered in its rotational freedom of any of the amino acids and may allow this region to move freely in the solvent, giving it maximum exposure to the protease.

The nicking of glutamine synthetase by proteases under nondenaturing conditions does not completely inactivate the enzyme. Nicked glutamine synthetase (GS*) catalyzes at least some of the same reactions as GS, but with a much lower efficiency than GS. A parallel decrease in activity with nicking is observed in the transferase and forward assays, indicating that adenylylated and unadenylylated subunits are cleaved at essentially the same rate. This observation is consistent with the observation of Lei et al. (1979) that the time course of nicking was the same for dodecamers that had states of ade-

Table II: Comparison of the Activities of GS and GS*

substrate	constant	value GS	value GS*
ATP	K_d, fluorescence (−Glu) (mM)	0.10[a]	0.14
	K_d, fluorescence (+Glu) (mM)	0.02[a]	0.18
	K_m, forward assay (mM)	1.13[c]	1.30
glutamate	K_d, calcd (−ATP) (mM)	20[b]	90
	K_m, forward assay (+ATP) (mM)	2.4[d]	90
FSO_2BzAdo	K_I, inactivation rate (mM)	0.23[c]	0.05
	K_i, competition with ATP (mM)	0.04[c]	0.05
	k_2, inactivation rate (min^{-1})	0.23[c]	0.02

[a] Slightly lower than similar values from *b*. [b] Timmons et al. (1974). [c] Foster et al. (1981). [d] Woolfolk et al. (1966).

nylylation ranging from 0.8 to 11.3.

It is possible that the activity of a preparation of nicked glutamine synthetase is due to the activity of a small amount of native GS. If this were the case, the relationship between activity and pH should be identical for GS and GS*. However, our results show that the pH optimum for the activity of both unadenylylated and adenylylated subunits in GS* was shifted 1.15 pH units when compared to GS. These data support the conclusion that the total activity of a preparation of nicked glutamine synthetase is not due to the contamination of GS* by GS. Further evidence of the activity of GS* comes from the substrate binding studies (Figures 3 and 4). The activity of nicked enzyme in the forward assay as a function of substrate concentration was determined to be due to two species of enzyme. One of the enzymes had the same Michaelis constant values for substrates as native GS (Table II): a K_m of 1.1 mM for ATP and 3 mM for glutamate. The other of these enzymes (GS*) had higher Michaelis constant values for substrates: a K_m of 1.3 mM for ATP and 80 mM for glutamate. From these data, we conclude that the activity of the native GS accounted for 10% of the total activity in a nicked preparation, corresponding to 0.1% of the total protein. Finally, a preparation of nicked enzyme was treated with methionine sulfoximine and ATP, which will interact with GS to form a tight, noncovalent complex that effectively inactivates the native enzyme. After treatment with methionine sulfoximine–ATP, the activity of the nicked enzyme was due only to GS*, as indicated by Michaelis contant values of 1.3 mM for ATP and 90 mM for glutamate.

The interaction of the ATP analogue 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine with GS* is somewhat different than the interaction of that analogue with GS. With GS, the K_I value for 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine is 0.22 mM and the k_2 value is 0.23 min^{-1}. For GS*, the values for K_I and k_2 were 0.05 mM and 0.023 min^{-1}, respectively, indicating that the binding of 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine to GS* is slightly tighter than the binding to GS, but the rate of inactivation of GS* is 10-fold slower than the rate of inactivation of GS. These results indicate that the portions of the enzyme that bind 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine are essentially unaffected by nicking. The terminal phosphate on ATP interacts with glutamate, possibly to form a γ-glutamyl phosphate derivative (Meister, 1974; Rhee & Chock, 1976). The fluorosulfonyl portion of 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine is in roughly the same position as the terminal phosphate of ATP (Pal et al., 1975). The portion of glutamine synthetase that controls interaction of the glutamate site with 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine has been altered by nicking; even though the analogue binds to GS* more tightly than to GS, the inactivation of GS* is 10-fold slower than the inactivation of GS. Also, with GS, glutamate enhanced the rate of inactivation of the enzyme by 5′-[*p*-(fluorosulfonyl)benzaoyl]adenosine (Foster et al., 1981). With GS*, glutamate had no effect on the rate of inactivation by the analogue.

Nicking of glutamine synthetase by proteases leads to the formation of a species of enzyme GS* that catalyzes some of the same reactions as GS, but with a lower efficiency. GS* binds ATP as well as GS but binds glutamate very poorly compared to GS. As indicated by the tight binding with a slower rate of inactivation by 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine, the interaction between the ATP and glutamate binding sites of GS* has been modified. Studies on GS* should be useful in determining the mechanism of action of GS, since the nicked enzyme is not susceptible to inactivation by methionine sulfoximine. Also, GS*, because of the apparent segregation of the ATP and glutamate sites, should be useful for determining the interaction of allosteric effectors with various portions of GS as well as clarifying the nature of some of the metal binding sites.

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Registry No. GS, 9023-70-5; FSO_2BzAdo, 57454-44-1; Met-(O)(NH), 1982-67-8; ATP, 56-65-5; glutamic acid, 56-86-0.

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pH Profiles and Isotope Effects for Aconitases from *Saccharomycopsis lipolytica*, Beef Heart, and Beef Liver. α-Methyl-*cis*-aconitate and *threo*-D_s-α-Methylisocitrate as Substrates†

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ABSTRACT: α-Methyl-*cis*-aconitate (*cis*-2-butene-1,2,3-tricarboxylate) was converted only to α-methylisocitrate (3-hydroxybutane-1,2,3-tricarboxylate) by aconitases from beef liver or *S. lipolytica*. While the kinetic parameters of beef liver (cytoplasmic) or heart (mitochondrial) aconitases did not vary over the pH range 4.9–9 with the natural substrates, and only slightly with the α-methyl substrates, the yeast aconitase exhibited a bell-shaped pH profile with all substrates and for binding of the competitive inhibitor, tricarballylate, with pK values around 7 and 9. The third pK of the substrates does not affect V/K, showing that these pK's are for catalytic groups on the enzyme. One of these catalytic groups presumably removes a proton to give the carbanion intermediate in the reaction, and the other protonates the hydroxyl group when it is eliminated to give water, possibly with the assistance of the Fe-S center. Beef liver aconitase showed a primary deuterium isotope effect of 1.12 (measured by equilibrium perturbation with deuterated α-methylisocitrate) which was pH independent and only slightly greater than the equilibrium isotope effect. Isotope effects with the yeast enzyme were also pH independent but about 1.22 on V/K (or when measured by equilibrium perturbation) and 1.7 on V. These data suggest a kinetic mechanism for beef aconitases in which product release occurs only by displacement by the substrate in a step independent of pH or of the protonation state of the substrate. With the yeast enzyme, product displacement either depends on the protonation state of the catalytic groups on the enzyme or can occur spontaneously at a finite rate. For all enzymes, binary complexes with reactants cannot have the catalytic groups incorrectly protonated.

Aconitase (EC 4.2.1.3), the second enzyme of the citric acid cycle, catalyzes the reversible hydration of *cis*-aconitate to either citrate or isocitrate. Because this reaction involves no net oxidation or reduction, the revelation that aconitase is an Fe-S protein (Kennedy et al., 1972; Suzuki et al., 1975b; Ruzicka & Beinert, 1978) came as a surprise. Further, aconitase loses activity on purification but can be activated with a reductant and Fe^{2+} (Dickman & Cloutier, 1950; Morrison, 1954; Villafranca & Mildvan, 1971; Gawron et al., 1974). These observations can now be explained by recent experiments which indicate that inactive aconitase contains a [3Fe-4S] cluster (Beinert et al., 1983) and that activation with iron and reductant converts this cluster to a [4Fe-4S] cluster (Kent et al., 1982; Kennedy et al., 1983). In addition, an intact [4Fe-4S] cluster in aconitase is required for full enzymatic activity (Emptage et al., 1983a).

Some insight into the potential role of the iron-sulfur cluster during catalysis has come from Mössbauer spectroscopy and perturbations in the EPR[1] spectra of the Fe-S cluster by

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[1] Abbreviations: EPR, electron paramagnetic resonance; Mes, 2-(*N*-morpholino)ethanesulfonate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonate; Tris, tris(hydroxymethyl)aminomethane; Caps, (cyclohexylamino)propanesulfonate; Ches, 2-(*N*-cyclohexylamino)ethanesulfonate; Taps, *N*-[tris(hydroxymethyl)methyl]glycine; SDS, sodium dodecyl sulfate; kDa, kilodaltons. $^{D}K_{eq}$, ^{D}V, $^{D}(V/K)$, and ^{D}(Eq.P.) represent deuterium isotope effects on K_{eq}, V, V/K, or one measured by equilibrium perturbation (that is, $K_{eq\,H}/K_{eq\,D}$, V_H/V_D, etc.).

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