Purification and Properties of the Phospho and Dephospho Forms of Yeast NAD-dependent Glutamate Dehydrogenase*

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The NAD-dependent glutamate dehydrogenase (GDH) was purified from *Candida utilis* and obtained in two molecular forms, dephosphoGDH (fully active) and phosphoGDH (less active). Both dephosphoGDH and phosphoGDH are homogenous according to the criteria of gel electrophoresis in the presence of sodium dodecyl sulfate and sedimentation in the ultracentrifuge. The two forms have a molecular weight of 460,000 ± 3,500 by sedimentation equilibrium, a $s_{20,w}$ of 14 S, and identical amino acid compositions, and are composed of four apparently identical subunits of 116,000 ± 2,000.

The phosphoGDH has about 1.2 mol of covalently bound phosphate/mol of subunit and an 8- to 10-fold reduced specific activity; dephosphoGDH has about 0.1 mol of covalently bound phosphate/mol of subunit. The phosphorylated amino acid was identified as serine. Kinetic analysis revealed similar Michaelis constants of 1.08 and 0.86 mM for NAD, but different affinities of 20 and 128 mM for L-glutamate for the dephospho and phospho forms of glutamate dehydrogenase, respectively. The two forms of the enzyme had different pH profiles when assayed in the reductive amination assay but did not differ significantly when assayed in the oxidative deamination assay.

Limited proteolysis of dephosphoGDH and phosphoGDH resulted in the production of two fragments of 64,500 and 48,000, suggesting that the enzyme is composed of two domains. The nicked enzyme remains tetrameric as judged by gel filtration studies. The proteolysis of phosphoGDH by trypsin produced a rapid reactivation of enzyme activity following by a loss of activity. Analysis of the products of limited proteolysis of $^{32}$P-labeled phosphoGDH showed that only the 48,000 fragment contained radioactivity. Prolonged trypsin treatment of dephosphoGDH resulted in a loss of enzyme activity.

Protein phosphorylation is recognized as one of the major mechanisms for controlling the catalytic activity of regulatory enzymes (for a review, see Krebs and Beavo (1)). Interconversion of enzymes between fully active and less active forms allows the flux of metabolites through a metabolic pathway to be finely controlled (2). The intricate cascade system elucidated from the study of regulation of glycogen synthesis and degradation is, perhaps, the best example of enzyme regulation by phosphorylation-dephosphorylation (3).

Regulation of enzyme activity by phosphorylation in yeast has received only scant attention. To date, the following proteins have been found to contain covalently attached phosphate: glycogen phosphorylase (4), glycogen synthetase (5), RNA polymerase (6), NAD-dependent glutamate dehydrogenase (7), and ribosomal proteins (8). Only in the cases of glycogen phosphorylase, glycogen synthetase, and glutamate dehydrogenase has the introduction of a phosphate group been shown to affect catalytic activity. The understanding of the significance of this type of covalent modification in a simple eukaryote is of interest from the point of view of both comparative biochemistry and evolution of regulatory systems. Additionally, the genetic manipulation of yeast offers a useful tool to elucidate the contribution of enzymes in covalent modification systems. In an earlier publication (9), the NAD-dependent glutamate dehydrogenase from *Candida utilis* was shown to be regulated phosphorylation promoted by the transition from glutamate-sufficient to glutamate-deficient culture conditions. The phosphorylation of NAD-dependent glutamate dehydrogenase is completely reversible in vivo and in vitro. The aim of the present work is to describe the basic molecular properties of the phospho and dephospho forms of the NAD-dependent glutamate dehydrogenase. This article reports the purification of NAD-dependent glutamate dehydrogenase, both in the dephosphorylated (active) and phosphorylated (partially active) states, referred to as dephosphoGDH and phosphoGDH, respectively.

Examination of the physicochemical and enzymatic properties of the two enzyme forms revealed that the introduction of a phosphate group results in a profound change in the catalytic properties ($V_{\text{max}}$, $K_m$ for glutamate, and pH profile) but does not affect the quaternary structure of the enzyme.

**Experimental Procedures**

Materials—DEAE-cellulose (DE52) was purchased from Whatman, Inc. Affigel-Blue-agarose and all electrophoresis chemicals were obtained from Bio-Rad Laboratories. Utrigel AcA34 and AcA22 were from LKB Instruments. Phenylmethylsulfonyl fluoride and pepstatin were purchased from Sigma Chemical Co. All nucleotides, α-ketoglutarate (disodium salt), and 5,5'-dithiobis(2-nitrobenzoic acid) were obtained from Boehringer. Coomassie blue G250 was obtained from Miles Laboratories.

Growth of *C. utilis*—*C. utilis* (NCYC 737) was grown in a 350- or 1100-liter fermentor on the medium described previously (9). For purification of dephosphoGDH, yeast were harvested at the end of log phase, frozen in liquid nitrogen, and stored at −20°C. For purification of phosphoGDH, yeast were harvested at the end of log phase and resuspended in the standard medium lacking glutamate for 2 h.

1 In an earlier publication (7), the two different forms of NAD-dependent glutamate dehydrogenase were referred to as GDHa (dephosphorylated) and GDHb (phosphorylated). With the mechanism of interconversion established, it is considered appropriate to refer to the two enzyme forms as dephosphoGDH and phosphoGDH. The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride, SDS, sodium dodecyl sulfate.
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After starvation, the cells were harvested and stored as described above.

To obtain cell-free extracts, yeast was passed six times through a Manton-Gaulin homogenizer (jacketed with ice water) at ~9000 p.s.i.

**Enzyme Assay**—The reductive amination assay previously described (9) was used to estimate specific activity, except that the total volume of the reaction mixture was increased to 2.0 ml. Typically, enzyme was assayed at pH 6.9 and 8.0 to determine the pH activity ratio (activity at pH 6.9/activity at pH 8.0). For fully dephosphorylated enzyme, this value is around 0.4 and, for the phosphorylated enzyme, it varies from 0.4 to 3.0 depending on the extent of phosphorylation.

For each experiment, the enzyme was assayed by oxidative deamination. The assay mixture (2.0 ml) contained 5 mM NAD and 300 mM monosodium glutamate; dissolved in 0.1 M Tris, pH 9.0. For pH profile studies, the monosodium glutamate (300 mM) was dissolved in 0.2 M Tris, adjusted to the desired pH with KOH or HCl, and diluted with H2O to give a final concentration of 0.1 M Tris. For kinetic investigations, the assay mixture was modified to contain the following: 25 mM Tris (pH 9.0), 150 mM KCl, 0.25 mM EDTA, and monosodium glutamate (adjusted to pH 9.0) and NAD at the desired final concentrations, in a total volume of 2.0 ml. For both assay systems, reactions were started by the addition of enzyme. The addition protocol to stabilize GDH during the assay, when low concentrations of glutamate were present.

**Protein Estimation**—In the early stages of developing the enzyme purification procedure, the method of Lowry et al. (10) was used with bovine serum albumin as a standard. This method was replaced by that of Bradford (11).

Protein concentration of the pure enzyme was determined using the methods of Lowry et al. (10) and Bradford (11) and by refractometry in the analytical ultracentrifuge (12). These three methods agreed to within 5%. The absorption coefficient, A280 nm, was determined at 9.06.

**Amino Acid Analysis**—Protein samples (0.8 mg) were hydrolyzed in 6 N HCl at 110°C for 24 h and 48 h, and 78 h. Samples were analyzed on a Beckman 120 C automated amino acid analyzer equipped with an integrator. Tryptophan was determined by amino acid analysis after hydrolysis with p-toluene sulfonic acid (13) and by second derivative spectrophotometry (14). This method was also used to determine tyrosine and phenylalanine. Total sulfhydryl groups were determined by titration, with 5,5'-dithiobis(2-nitrobenzoic acid) on sodium dodecyl sulfate denatured enzyme (15).

**Alkalai-labile Phosphate Analysis**—Determination of phosphate covalently bound to NAD-dependent glutamate dehydrogenase was performed as described by Nimmo et al. (15) according to the method of Ames (16). Purified enzyme preparations were dialyzed against 50 mM Tris, 200 mM KCl, 1 mM EDTA, 2 mM MgCl2, and 2 mM phenylmethylsulfonyl fluoride, pH 7.0 (3 x 6 liters), for 24 h at 4°C. After dialysis, any denatured protein was removed by centrifugation. Enzyme and protein concentrations were determined. Protein samples (2 to 4 mg) were then precipitated by the addition of trichloroacetic acid to a final concentration of 5%. The protein was then processed exactly as described by Nimmo et al. (15). For the calculations of the stoichiometry of bound phosphate, the subunit molecular weight was taken as 110,000. Ovalbumin was used as control protein and by this method contained 2 molecules of phosphate-subunit ([M] = 45,000). With this method of phosphate analysis, 10 nmol gives an A280 of 0.25.

**Identification of [32P]Phosphoserine**—32P-Labeled phosphoGDH (1 mg) was hydrolyzed in 0.5 ml of 5.7 N HCl for 5 h at 110°C or 5 min at 155°C (17). The hydrolysate was lyophilized, dissolved in 10 mM HCl with 0.5 mg of pure phosphoserine and phosphothreonine, and applied to a Dowex AG 50W-12 column (1 x 25 cm) equilibrated with 10 mM HCl (18). The flow rate was 12 ml/h; 1-ml fractions were collected and analyzed for radioactivity and for ninhydrin positive material.

**Gel Electrophoresis**—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (19). The following proteins were used as standards: myosin (220,000), RNA polymerase (160,000), β-galactosidase (116,000), phosphorylase (94,000), bovine serum albumin (68,000), ovalbumin (45,000), soybean trypsin inhibitor (21,500), and lysozyme (13,500).

**Sedimentation Equilibrium**—Sedimentation coefficients were performed at 20°C with a Beckman model E analytical ultracentrifuge equipped with a rotor temperature and control unit) using schlieren optics with a plate phase plate and a speed of 48,000 rpm. Instrument calibrations, sequence photographs, and density and viscosity measurements were the same as those described by Shapiro and Ginsberg (20). A two-place AnD rotor and two cells containing 12-mm, 4° single sector Kel F centerpieces were used for measurement of ΔΔc of phosphoGDH with protein concentrations of 2.0 and 7.8 mg/ml; one of the two cells had a 1° positive wedge quartz upper window for orientation. A computer program was used for the calculation of sedimentation coefficient of phosphoGDH. A Kel F double sector centerpiece was used for sedimentation experiments with phosphoGDH (1.9 and 2.9 mg/ml).

Before ultracentrifugation, samples of phospho- or dephosphoGDH were dialyzed at 4°C for 24 h against 0.1 M NaPO4 buffer (pH 7.5) containing 1 mM sodium EDTA (buffer density at 20°C = 1.012 g/ml and relative viscosity at 25°C = 1.062). Dilutions of dialyzed enzyme solutions were made with the corresponding dialysate (buffer). Measured sedimentation coefficients were corrected to values (S20, w) corresponding to a solvent with a viscosity and density of water at 20°C.

Sedimentation equilibrium studies were performed in the laboratory of Dr. Marc S. Lewis (Laboratory of Biomedical Engineering and Instrumentation, National Institutes of Health) using a Beckman model E analytical ultracentrifuge equipped with a Multiplex helium Noble liquid light source with a Rayleigh interference optical system. A 12-mm cell with sapphire windows and a carbon-filled epoxide capillary boundary forming centimeter was used; buffer was layered on top of 0.05 ml of protein solution (1.3 mg/ml) by running at 20,000 rpm for 10 min. The rotor speed was then reduced to 6,000 rpm and controlled at this speed for 48 h and 20°C. Interference patterns were photographed on Kodak X-OMAT high-speed aerial photographic film. The films were read on a Nikon 6C comparator equipped with digital readout lead screws and photoelectric fringe center locator. Data was analyzed using MLAB interactive system for mathematical modeling operating on the National Institutes of Health DEC system-10 computer by fitting the data to the equation for concentration distribution in the ultracentrifuge cell c = c0 exp (Mw, app x [r2 - r02]) where A = (1 - Vr) w2(R2 - r02). c is the concentration at radius r and c0 is the concentration at radius r0. Mw, app and c0 are then obtained as fitting parameters. Mw, app is the apparent weight average molecular weight of the macromolecular species, V is the partial specific volume, ρ is the solution density, w is the angular rotor speed, R is the gas constant, and T is the absolute temperature.

**RESULTS**

**Purification of Yeast NAD-dependent Glutamate Dehydrogenase**—All steps were performed at 4°C. Routinely, 1 to 2 mM phenylmethylsulfonyl fluoride (PMSF) was added to all buffers. The problem of proteases contaminating the glutamate dehydrogenase preparations was most pronounced after the affinity chromatography step.

**Purification of DephosphoGDH**—About 1 kg of yeast was resuspended into 2000 ml of 100 mM sodium phosphate at pH 7.5 containing 5 mM EDTA, 27 mM glutamate (potassium salt) and PMSF and then passed six times through a Manton-Gaulin mill (at 9000 p.s.i.) to ensure complete cell disruption. The broken cell suspension was centrifuged for 1 h at 16,000 × g. The resulting supernatant is referred to as the "crude extract."

The crude extract was brought to 27.5% saturation by slow addition of solid (NH4)2SO4 with continuous stirring. After 60 min, the suspension was centrifuged (30 min at 16,000 × g) and the supernatant solution was taken to 60% saturation in (NH4)2SO4. After 60 min, the mixture was centrifuged at 19,000 × g for 50 min. The pellet containing most of the enzyme activity was resuspended into a minimal volume of 20 mM sodium phosphate, pH 7.5, containing 5 mM EDTA, 5 mM α-ketoglutarate, 0.1 mM NAD and PMSF and dialyzed overnight against three changes of the same buffer (3 x 6 liters). The dialysate was centrifuged for 60 min at 19,000 × g and the clear supernatant solution was chromatographed on a column (5 x 30 cm) of DEAE-cellulose (Pharmacia DE52). After washing the column with 300 ml of equilibrating buffer, the enzyme was eluted with a 4-liter gradient of NH4Cl from 0 to 0.4 M in the equilibrating buffer. The fractions containing

2R. L. Levine and M. M. Federici, unpublished observations.
glutamate dehydrogenase activity were pooled and concentrated by ultrafiltration in an Amicon pressure unit. The concentrated fraction was then passed over a hemoglobin-glutamate dehydrogenase column in a small Buchler funnel to remove proteases.

The enzyme was then applied to an Affigel-Blue-agarose column (6.5 x 4 cm) equilibrated with 100 mM sodium phosphate, pH 7.5, containing 100 mM KCl, 5 mM EDTA, 5 mM PMSF, and 1 mM NADH. The NAD-dependent glutamate dehydrogenase activity was pooled and concentrated by ultrafiltration in an Amicon pressure unit (XM 50) and then applied to a column (5 x 50 cm) of Ultrogel AcA22 previously equilibrated with 100 mM sodium phosphate, pH 7.5, containing 100 mM KCl, 5 mM EDTA, 5 mM α-ketoglutarate, 0.2 mM NAD, and 1 mM PMSF. The column was eluted at a flow rate of 60 ml/h and fractions of 5 ml were collected as shown in Fig. 1. In some preparations, it was necessary to pass the enzyme over a second gel filtration column to obtain pure enzyme. Fractions with the highest specific activity were pooled and stored as a suspension in 55% saturated ammonium sulfate as 4°C. The purification procedure was summarized in Table I.

**Purification of PhosphoGDH**—To minimize reactivation (dephosphorylation) of phosphoGDH in crude extracts, the purification procedure was modified. Yeast (300 g) were resuspended in 2 liters of 20 mM sodium phosphate, pH 7.5, containing 5 mM EDTA and then passed six times through a Manton-Gaulin and centrifuged for 30 min at 16,000 x g. The resulting clear supernatant was added directly to 200 g of DEAE-cellulose previously equilibrated with the above buffer and stirred for 1 h. It was found that the reacting factor, presumably a phosphatase, eluted from the DEAE-cellulose column at a higher ionic strength than phosphoGDH. Thus, the enzyme could be maintained in the inactive state by rapidly applying crude extracts to the ion exchange column. However, in large scale purifications, some reactivation of the enzyme occurs during cell disruption and centrifugation.
Phospho and Dephospho Forms of Yeast Glutamate Dehydrogenase

The molecular weight of the NAD-dependent glutamate dehydrogenase subunit determined by SDS-polyacrylamide gel electrophoresis was 116,000 ± 3,000 in 10% and 7.5% gels. Both dephospho- and phosphoGDH had identical mobilities to the E. coli β-galactosidase (Mr = 116,000). The molecular weight values for the subunit and native enzyme indicate that the NAD-dependent glutamate dehydrogenase is a tetrameric protein.

The absorbance coefficient of dephosphoGDH, A280 nm, is 9.06 in 0.1 M sodium phosphate, pH 7.5, and 1 mM EDTA (calculated from the protein concentration determined in the analytical ultracentrifuge by the interference optics method of Babul and Stellwagen (12)). The ratio of the absorbance of the enzyme at 280 nm to its absorbance at 290 was 1.90.

**Amino Acid Analysis**—The amino acid composition for both dephosphoGDH and phosphoGDH is given in Table III. Numbers of residues were calculated for a subunit of molecular weight of 116,000. Values presented for dephosphoGDH are the averages obtained from three times of hydrolysis on duplicate samples using two different batches of purified enzyme. Amino acid composition of phosphoGDH was determined with a single batch of protein.

Also listed in Table III is the amino acid composition of the NAD-dependent glutamate dehydrogenase from Neurospora crassa (taken from Veronese et al. (24)).

**Content of Phosphate Bound Covalently to DephosphoGDH and PhosphoGDH**—A number of samples of dephospho- and phosphoGDH were analyzed for alkali-labile phosphate (Table IV). The results show that dephosphoGDH contained a small amount of phosphate, about 0.1 mol/mol of subunit. PhosphoGDH, on the other hand, contained just over 1 mol of phosphate/mol of subunit. The difference in the specific activity of the two enzyme forms from this data is about 8- to 10-fold. At this moment, we are unable to describe exactly the correlation between enzyme activity, pH ratio change, and the extent of phosphorylation with the data available, although the limited data available suggest a linear relationship between extent of phosphorylation and activity.
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The phosphate group bound to phosphoGDH was acid-stable and alkali-labile; incubation of 32P-labeled phosphoGDH at 90°C for 10 min in 1 N NaOH hydrolyzed up to 90% of the radioactivity. Other experiments showed the phosphorylated amino acid to be serine. Chromatography of acid-hydrolyzed 32P-labeled enzyme on Dowex AG 50W-X12 yielded two peaks of radioactivity which were identified as free phosphate and phosphoserine.

pH Optima of DephosphoGDH and PhosphoGDH—Experiments carried out using crude extracts showed the NAD-dependent glutamate dehydrogenase to have a different pH optimum after in vivo inactivation, when assayed using the reductive amination system. These results were confirmed using purified preparations of dephospho- and phosphoGDH (Fig. 3). These results show the pH activity profile of four different enzyme preparations which contained different amounts of covalently bound phosphate. The fully active enzyme has optimal activity at pH 8.1. The enzyme preparation with 75% of full activity (0.86 mol of phosphate/mol of subunit) showed an optimum at a slightly more acidic value (7.9 to 8.0), whereas the activity profiles of the two phosphoGDH preparations which contain about 1.2 mol of phosphate/mol of subunit differ significantly from dephosphoGDH and both show optimal activity at pH 7.0.

In the direction of glutamate oxidation, dephosphoGDH had optimal activity at pH 9.4 (Fig. 3A). The optimum for phosphoGDH is less clearly defined due to its low activity, but maximal activity is observed around pH 9.4 (Fig. 3B).

Kinetic Parameters—The kinetic parameters for NAD and glutamate were determined, at pH 9.0, according to Cleland (25, 26). This procedure gives both the true Vmax and the true Km for a bisubstrate reaction. The values for the two enzyme forms are shown in Table V. These data illustrate that phosphorylation of glutamate dehydrogenase results in a decrease of Vmax and a decrease in affinity for glutamate. Both of these changes in the properties of the enzyme would probably act in concert to make the phosphorylated enzyme totally inactive in vitro. This is illustrated in Fig. 4, where initial velocity of the dephospho- and phosphoGDH are shown for varying glutamate concentrations (with NAD held constant). Thus, in the physiological range of concentration for glutamate, the difference in the specific activities of the two forms of the enzyme would be even greater (up to 20-fold).

The phosphoGDH used in these studies was not in the fully phosphorylated state because during the initial stages of enzyme isolation some reactivation (dephosphorylation) of enzyme activity is observed. Thus, it is conceivable that the differences between the dephospho and phosphoenzyme forms are even greater. Confirmation of this suggestion will be obtained when it is possible to purify fully phosphorylated enzyme or fully phosphorylate the enzyme in vitro.

Effect of Trypsin on DephosphoGDH and PhosphoGDH—In an earlier communication, it was reported that trypsin could be used to reactivate phosphoGDH in crude extracts (9). This result has been confirmed and extended using pure preparations of phosphoGDH.

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TABLE V

Kinetic parameters of NAD-dependent glutamate dehydrogenase

The kinetic parameters for NAD and L-glutamate (Na+) were determined according to Cleland (25, 26). All data were fitted by the method of least squares.

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Fig. 3 (left and center). Effect of pH and extent of phosphorylation on enzyme activity. A, effect of pH and extent of phosphorylation on the reductive amination assay of glutamate dehydrogenase. The four curves are those obtained from four different purified preparations of glutamate dehydrogenase, containing 0.092 mol of P/mol of subunit (O), 0.87 mol of P/mol of subunit (O), 1.19 mol of P/mol of subunit (O), and 1.23 mol of P/mol of subunit (O), respectively. B, effect of pH and extent of phosphorylation on the oxidative deamination assay of glutamate dehydrogenase. Two enzyme preparations were used containing 0.09 mol of P/mol of subunit (O) and 1.19 mol of P/mol of subunit (O), respectively.

Fig. 4 (right). Kinetics of initial reaction rates of dephosphoGDH (O) and phosphoGDH (O) as a function of glutamate concentration. Rates are expressed as specific activities so that a direct comparison of the activity of the two forms can be made. The reactions were carried out in 0.1 M sodium pyrophosphate, pH 9.0, buffer at 30°C and started by the addition of enzyme. NAD concentration was held constant at 2.1 mM.
Glutamate dehydrogenase activity was measured at pH and phosphoGDH with symbols phosphoGDH, a-ketoglutarate, 10 mM MgSO₄, 10 mM oxidized glutathione, and 4 mM NAD; closed symbols are incubation mixtures with the addition of effectors. O, no trypsin; □, 5 µg/ml of trypsin; △, ▲, 50 µg/ml of trypsin. B, phosphoGDH (0.48 mg/ml) was incubated with trypsin in 0.1 M sodium phosphate, pH 7.5, 5 mM EDTA, and 0.1 mM NAD at 30°C. Symbols are the same as those described for A except that the final concentration of trypsin was 25 µg/ml (□, ■) and 100 µg/ml (△, ▲).

Incubation of purified phosphoGDH with trypsin promoted a rapid increase of enzyme activity (Fig. 5A). Prolonged incubation in the presence of large amounts of trypsin (50 µg/ml) caused a subsequent loss of enzyme activity. Addition of NADH, a-ketoglutarate, Mg²⁺, and oxidized glutathione to the incubation mixture stimulated the rate of reactivation and also stabilized the enzyme against loss of activity during prolonged incubation with trypsin. During studies of a yeast "reactivating activity" on phosphoGDH, the combination of these effectors were found to stimulate in vitro reactivation (27). Subsequently, they were tested with trypsin and found to have a similar effect. The mechanism of the interaction of these compounds on the reactivation is unknown.

The effect of trypsin on dephosphoCDH was also examined (Fig. 5B). Proteolytic action of trypsin on dephosphoCDH caused a time-dependent loss of enzyme activity. Incubation of dephosphoCDH with NADH, a-ketoglutarate, Mg²⁺, and oxidized glutathione stabilized the enzyme against the effect of trypsin.

The action of trypsin did not cause a detectable change in the molecular weight of either enzyme form as judged by gel filtration studies. Analysis of the products of limited proteolysis by trypsin on SDS-polyacrylamide gels showed, for both forms of the enzyme, that the initial major fragments produced had molecular weights of 64,500 and 48,000 (Fig. 6). These experiments were repeated using ³²P-labeled phosphoGDH. Treatment of phosphoGDH with trypsin produced the two major fragments; however, after reactivation, all the radioactivity formerly associated with the native subunit of 116,000 was found to be attached to the 48,000 fragment. Prolonged incubation of the enzyme produced a second radioactive fragment which migrated with the dye front on 10% polyacrylamide gels, i.e. a polypeptide with a molecular weight of less than 10,000.

Limited digestion of dephospho- or phosphoGDH by chymotrypsin also generated two major fragments with molecular weights of 65,000 and 45,000.

**Immonochemical Properties**—Antibodies were prepared to dephosphoGDH. These antibodies reacted equally well with either enzyme form by the Ouchterlony double diffusion procedure; a continuous precipitin band formed without any spurs. The antibodies did not cross-react with pure NADP-dependent glutamate dehydrogenase from either C. utilis or Saccharomyces cerevisiae, or with bovine glutamate dehydro-
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The data presented demonstrate that the NAD-dependent glutamate dehydrogenase from C. utilis is quite unique compared to glutamate dehydrogenases isolated from other sources. The C. utilis enzyme is a tetramer composed of four apparently identical subunits which have a molecular weight of 116,000, and its activity is regulated by a phosphorylation-dephosphorylation system. The glutamate dehydrogenases isolated from most other sources are hexameric proteins with subunit molecular weight of around 50,000 and activity regulation is achieved via allosteric effectors (28). The only other exception is the NAD-dependent glutamate dehydrogenase isolated from N. crassa, which is also a tetrameric protein composed of four subunits of 116,000 (24). It is not known whether the enzyme from N. crassa is regulated by covalent modification.

The purification schemes outlined for dephospho- and phospho-enzyme are convenient and allow the isolation of large quantities of protein in 1 week. However, extreme precautions are necessary to avoid proteolysis of the enzyme, especially after the Affigel-Blue-agarose step. In recent work, we have used hemoglobin-agarose columns to remove protease contamination from the glutamate dehydrogenase preparations, usually before the affinity chromatography step. The isolation of the phosphorylated form of glutamate dehydrogenase, however, is more problematic because of the action of phosphatases and, if precautions are not taken, proteases. Both of these enzymes lead to reactivation of the phosphoGDH either by dephosphorylation or limited proteolysis. Attempts to isolate the phosphoGDH by a similar method as that described for dephosphoGDH led to dephosphorylation and reactivation of the glutamate dehydrogenase during dialysis prior to DEAE-cellulose chromatography. Loading of crude extracts directly to DEAE-cellulose allows the isolation of phosphoGDH free of phosphatase. (The phosphatase activity elutes at a higher ionic strength than the glutamate dehydrogenase.) After this initial step, the phosphoGDH was found to be quite stable and not reactivated during subsequent purification procedures.

The specific activity of the dephosphoGDH was around 450 units/mg of protein, which compares with that obtained by Veronese et al. (24) for the NAD-dependent glutamate dehydrogenase from N. crassa. The lowest specific activity obtained with any preparation of the purified phosphoGDH was 50 units/mg. Other experiments with crude extracts indicate that the maximum difference in specific activities between the two forms of enzyme is around 5-fold (when assayed at pH 8.0 using the reductive amination method). All preparations of phosphorylated glutamate dehydrogenase contained slightly more than 1 mol of phosphate/mol of subunit. Therefore, it is possible that in the fully inactive state the phosphoGDH could contain up to 2 mol of phosphate/mol of subunit.

The molecular weight of both enzyme forms was found to be around 480,000 by sedimentation equilibrium studies. This is slightly lower than that obtained for the N. crassa enzyme. From sedimentation velocity experiments, a sedimentation coefficient of 14.0 S was found. No evidence for association-dissociation was observed with either method.

Gel filtration chromatography of dephospho- and phosphoGDH produced slightly anomalous results inasmuch as the enzyme had an apparent molecular weight of around 800,000 under several different conditions. At this moment, we have no explanation for the behavior of the enzyme during gel filtration studies.

A value of 116,000 was estimated for the subunit molecular weight by SDS-polyacrylamide gel electrophoresis; therefore, the NAD-dependent glutamate dehydrogenase from C. utilis would appear to be a tetramer of apparently identical subunits. Recent experiments with the enzyme purified from S. cerevisiae indicate that the NAD-dependent glutamate dehydrogenase from this organism also has a subunit with a molecular weight of around 116,000. The enzyme from this organism also undergoes rapid activity modulation which appears to be mediated by phosphorylation.¹ So it appears generally that the NAD-dependent glutamate dehydrogenase from fungi and yeast are considerably different from other glutamate dehydrogenases (see Smith et al. (28) for a review) which have subunit sizes of about 50,000 and exist as hexamers. Smith (29) has suggested that the NAD-dependent glutamate dehydrogenase has a distant evolutionary relationship to other glutamate dehydrogenases. It would be interesting to know whether this divergence was necessary to accommodate regulation of enzyme activity by phosphorylation-dephosphorylation or vice versa. It has been proposed that the large subunit of the NAD-dependent glutamate dehydrogenase results from gene fusion (29).

The studies of the limited proteolysis of GDH offer a possible explanation to the evolution of the NAD-GDH. Haberland and Smith (30) have conducted similar tryptic digests of the NAD-GDH from N. crassa. Limited proteolysis resulted in the 116,000 subunit being converted to fragments of 47,300 and 63,100 corresponding to the NH₂- and COOH-terminal portions of the enzyme, respectively. The 63,100 fragment was sequenced and found to contain considerable homology to the amino acid sequences determined for GDH from other sources. It is thought that the 63,100 fragment from N. crassa enzyme corresponds to the catalytic domain (30). From the data obtained with ³²P-labeled NAD-GDH from C. utilis, the smaller tryptic fragment (48,000) was shown to contain the phosphorylation site and as a consequence probably corresponds to a regulatory domain. The fact that the enzyme can be reactivated by limited proteolysis suggests that the 48,000 fragment domain interacts with the 65,000 domain and, when it is phosphorylated, results in enzyme inactivation. Reactivation of phosphoGDH by trypsin is probably mediated by conformational change following cleavage which decreases the interaction of the phosphorylated regulatory domain with the catalytic domain, resulting in the relief of inhibition of the active site.

Amino acid analysis (Table III) confirms the similarity between the NAD-dependent glutamate dehydrogenase from C. utilis and the enzyme from N. crassa.

The data presented here and that in an earlier publication (7) confirm that the activity of the NAD-dependent glutamate dehydrogenase from C. utilis is regulated by phosphorylation. Enzyme isolated from glutamate-sufficient cultures contains little or no alkali-labile phosphate, whereas enzyme purified from glutamate-starved cultures contains about 1.2 molecules of phosphate/subunit. Thus, glutamate starvation promotes the rapid loss of enzyme activity by the introduction of phosphate. Experiments with ³²P-labeled phosphoGDH showed that the phosphate is covalently attached to a serine residue(s). In an earlier publication (7), it was demonstrated that dephosphorylation of phosphoGDH resulted in a rapid reactivation of enzyme activity.

Comparison of the properties of dephospho- and phosphoGDH revealed that phosphorylation resulted in changes.

¹ B. Hemmings, unpublished data.
in enzymatic properties, the most striking being differences in $V_{\text{max}}$, the $K_m$ for L-glutamate, and the pH profile for reductive amination. The reduction of both $V_{\text{max}}$ and affinity for L-glutamate following the introduction of phosphate probably renders the glutamate dehydrogenase inactive in vivo, thereby stopping the depletion of the intracellular pool of glutamic acid.

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