The active NAD-dependent glutamate dehydrogenase of wild type yeast cells fractionated by DEAE-Sephacel chromatography was inactivated in vitro by the addition of either the cAMP-dependent or cAMP-independent protein kinases obtained from wild type cells. cAMP-dependent inhibition of glutamate dehydrogenase activity was not observed in the crude extract of byc1 mutant cells which were deficient in the regulatory subunit of cAMP-dependent protein kinase. The cAMP-dependent protein kinase of CYR3 mutant cells, which has a high $K_v$ value for cAMP, proved to be active in the phosphorylation reaction, required a high cAMP concentration for the inactivation of NAD-dependent glutamate dehydrogenase. An increased inactivation of partially purified active NAD-dependent glutamate dehydrogenase ($M_\text{r} = 450,000$) was observed to correlate with increased phosphorylation of a protein subunit ($M_\text{r} = 100,000$) of glutamate dehydrogenase. The phosphorylated protein was labeled by an NADH analog, 5'-p-fluorosulfonyl-14Cbenzoyladenosine. Activation and dephosphorylation of inactive NAD-dependent glutamate dehydrogenase fractions were observed in vitro by treatment with bovine alkaline phosphatase or crude yeast cell extracts. These results suggested that the conversion of the active form of NAD-dependent glutamate dehydrogenase to an inactive form is regulated by phosphorylation through cAMP-dependent and cAMP-independent protein kinases.

It is known that the activities of some enzymes are regulated by a phosphorylation-dephosphorylation mechanism and that the reactions involved in the phosphorylation and dephosphorylation of proteins are catalyzed by protein kinases and phosphoprotein phosphatases (1). Yeast cells contain at least two types of protein kinases, cAMP-dependent and cAMP-independent (2, 3). The activation of trehalase in Saccharomyces cerevisiae has been known to be regulated by the cellular levels of cAMP (4-7), and recently we have indicated that the conversion of the inactive form of trehalase to an active form is catalyzed by cAMP-dependent protein kinase (8). In Candida utilis, the phosphorylation of NAD-dependent glutamate dehydrogenase resulted in the conversion of the active enzyme form to a less active enzyme form, and this conversion of the enzyme form was reversible in vivo and in vitro (9-12).

The study on in vivo roles of enzymes may be greatly facilitated by isolation of mutants defective in the enzyme activity. Recently, we isolated several yeast mutants which were altered in adenylate cyclase or cAMP-dependent protein kinase (3, 13, 14). Among them, the byc1 mutant lacked cAMP-dependent protein kinase, but produced a high level of cAMP-independent protein kinase (3), and the CYR3 mutant produced altered cAMP-dependent protein kinase which had significantly high $K_v$ values for cAMP (13). Using these mutants, we show that yeast NAD-dependent glutamate dehydrogenase is inactivated through phosphorylation by cAMP-dependent and cAMP-independent protein kinases and is reversibly activated by dephosphorylation.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains**—The wild type strain (F10D) of S. cerevisiae was our stock culture (15). All other strains were derived from the same wild type strain as described in our previous papers (3, 13, 14).

**Culture**—Yeast cells were grown in a medium containing 10 g of yeast extract, 20 g of Polypeptone and 20 g of glucose/liter.

**Preparation of Cell-free Extract**—All experiments were carried out at 4°C. Cells were harvested by centrifugation, washed well with distilled water, and suspended in a buffer containing 50 mM Tris-HCl buffer (pH 7.4), 1 mM EDTA, 1 mM 3-mercaptopethanol and 0.5 mM phenylmethylsulfonyl fluoride (buffer T). The cell suspension was disrupted with an Amino French pressure cell at 10,000 p.s.i. The resulting homogenate was centrifuged at 1,000 $X$ g for 10 min. The supernatant fluid was used as a crude extract. The 20,000 $X$ g supernatant fluid was obtained by centrifuging the crude extract at 20,000 $X$ g for 30 min.

NAD-dependent Glutamate Dehydrogenase Assay—NAD-dependent glutamate dehydrogenase activity was measured as follows; the reaction mixture (final volume, 5.0 ml) contained 20 mM a-ketoglutarate, 40 mM NH$_4$Cl, 0.2 mM NADH, 80 mM Tris-HCl buffer (pH 9.0), and enzyme solution. The reaction was initiated by the addition of NADH, and the absorbance at 340 nm was monitored. One unit of enzyme activity was defined as the amount of enzyme which produced 1 pmol of glutamate in 1 min at 30°C in the standard assay method.

**NAD-dependent Glutamate Dehydrogenase Inactivation**—When crude extract was used, it was preincubated with 0.1 mM ATP in the absence or presence of 10 mM cAMP at 30°C for 5 min and adequately diluted with buffer T. The diluted preparation was assayed for glutamate dehydrogenase activity. Partially purified glutamate dehydrogenase was preincubated in a final volume of 100 pl with 0.1 unit of cAMP-dependent protein kinase partially purified from wild type cells (F10D) through DEAE-Sephacel chromatography and 0.1 mM ATP in the absence or presence of 10 mM cAMP at 30°C for 5 min. The preparation was diluted at least 25-fold and then assayed for glutamate dehydrogenase activity.

**Protein Kinase Assay**—Protein kinase activity was measured by the method described previously (16). The standard reaction mixture (final volume, 50 pl) contained 50 mM Tris-HCl buffer (pH 7.4), 80 $\mu$g of type II histone, 5 mM MgCl$_2$, 5 $\mu$M [y-32P]ATP (1 x 10^4 dpm/mmoll), 10 $\mu$M cAMP, and 20 $\mu$l of enzyme solution. The reaction was initiated by adding 10 $\mu$l of [y-32P]ATP and terminated by transferring a 25-$\mu$l aliquot of the reaction mixture to a section of phosphocellulose paper (1 x 2 cm) which was immersed in a beaker containing distilled water. The sample was washed with distilled water and acetone. After drying in air, the radioactivity adsorbed onto the phosphocellulose paper was measured by a liquid scintillation spectrometer. One unit of enzyme activity was defined as the amount of...
enzyme which transferred 1 pmol of \([\gamma-3^P]ATP\) to recovered protein in 1 min at 30 °C in the standard assay system.

Labeling of NAD-dependent Glutamate Dehydrogenase by 5'-p-Fluorosulfonyl[14C]benzoyladenosine—Labeling experiments were performed as described by Pal et al. (17). The standard reaction mixture (final volume, 100 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM 5'-p-fluorosulfonyl[14C]benzoyladenosine (50 mCi/mmol), and various amounts of protein. Incubation was carried out for 60 min at 35 °C, and the reaction was quenched by the addition of 50 μl of the stop solution containing 30 mM Tri-HCl buffer (pH 7.4), 3 mM EDTA, 9% glycerol and 0.56 mM β-mercaptoethanol. The solution was applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the labeled protein was detected by fluorography, and the representative channels of dried gels were sliced and radioactivity of each slice was counted by a liquid scintillation spectrometer.

Protein Phosphorylation—Protein phosphorylation was carried out as follows. The standard reaction mixture (final volume, 100 ml) contained 50 mM, 1.4-piperazinediethanesulfonic acid buffer (pH 7.0), 5 mM MgCl₂, 4 μM \([\gamma-3^P]ATP\) (12 Ci/mmol), 0.21 unit of cAMP-dependent protein kinase, and 70 μl of sample in the absence or presence of 10 μM cAMP. The reaction was initiated by the addition of the enzyme which transferred 1 pmol of \([\gamma-3^P]ATP\) to recovered protein

Protein Measurement—Protein was measured by the method of Lowry et al. (18) using bovine albumin as the standard.

DEAE-Sepahcel Chromatography—A 20,000 X g supernatant fluid (10–50 ml) was applied to a DEAE-Sepahcel column (2.0 x 20 cm) which had been equilibrated with buffer T. Proteins were eluted from the column with a linear gradient (0–0.5 M) of NaCl in a total volume of 200–400 ml.

Sephadex G6 Gel Filtration—One ml of partially purified active NAD-dependent glutamate dehydrogenase was applied to a Sephadex G6 column (1.5 x 40 cm) which had been equilibrated with buffer T. NAD-dependent glutamate dehydrogenase was eluted with the same buffer.

Chemicals—\([\gamma-3^P]ATP\) (3,000 Ci/mmol) was purchased from Amersham. 5'-p-Fluorosulfonyl[14C]benzoyladenosine from New England Nuclear; ATP, CAMP, 5'-adenylylimidodiphosphate, alkaline phosphatase (type I from bovine intestine), and histone (type II) from Sigma; DEAE-Sepahcel and Sephacel G6 from Pharmacia; phosphocellulose paper (P81) from Whatman; molecular weight markers from BDH; Polypeptone from Wako Chem. Co.; and yeast extract from Difco Laboratories.

RESULTS

Inactivation of NAD-dependent Glutamate Dehydrogenase—NAD-dependent glutamate dehydrogenase activity in crude extracts prepared from wild type (F10D) cells decreased to about 15% of the original activity by preincubation with increasing amounts of ATP (Fig. 1). When 10 μM of cAMP was supplemented in addition to ATP, a drastic inhibition of the enzyme activity was observed even with 0.1 mM ATP (Fig. 1). The inactivation reached a maximal level within 4 min under the present condition. An ATP analog, 5'-adenylylimidodiphosphate, could not inhibit glutamate dehydrogenase activity even in the presence of cAMP.

NAD-dependent glutamate dehydrogenase activity in crude extracts of bcy1 mutant (AM27-2B) cells decreased to about 15% of the original activity by preincubation with 1.0 mM ATP at 30 °C for 5 min, but in contrast to the wild type enzyme, no further inhibition of the enzyme activity was observed by the addition of 10 μM cAMP (Fig. 1). The bcy1 mutant cells are known to have no cAMP-dependent protein kinase, but produce a high level of cAMP-independent protein kinase (3). A partial inactivation of glutamate dehydrogenase activity by preincubation with 10 μM cAMP and 0.1 mM ATP was observed in the crude extract of CYR3-1 mutant (AM21-2A) cells (Fig. 1). It has been shown in our previous studies that the cAMP-dependent protein kinase activity of CYR3-1 cells showed significantly higher K₅ values for cAMP than those of wild type cells (13). These results suggest that the significant inactivation of NAD-dependent glutamate dehydrogenase found in the presence of cAMP and ATP may be a consequence of cAMP-dependent phosphorylation of enzyme protein, and the partial inactivation found with higher concentrations of ATP in the absence of cAMP may be due to cAMP-independent phosphorylation.

Identification of Glutamate Dehydrogenase-inactivating Factors—To test the possibility that protein kinase is responsible for the inactivation of NAD-dependent glutamate dehydrogenase, crude extract of the wild type strain was applied to a DEAE-Sepahcel column and eluted with a linear gradient of NaCl (Fig. 2). One peak of glutamate dehydrogenase activity was observed on the chromatogram. The glutamate dehydrogenase activity in the peak fractions could not be inhibited by adding cAMP and ATP. The peak fractions of glutamate dehydrogenase activity were pooled, concentrated by ultrafiltration, and used to assay the glutamate dehydrogenase-inactivating activity. When aliquots of the pooled enzyme sample were added to all fractions eluted from the DEAE-Sepahcel column, two troughs of glutamate dehydrogenase-inactivating activity were found on the chromatogram (Fig. 2). Inhibition of glutamate dehydrogenase activity at the first trough was stimulated remarkably by preincubation with 10 μM cAMP and 0.1 mM ATP and that at the second trough was not influenced by the presence of cAMP (Fig. 2). These troughs of glutamate dehydrogenase-inactivating activity were found at exactly the same positions as peaks of cAMP-dependent and cAMP-independent protein kinases as shown in Fig. 2. These results indicate that cAMP-dependent and cAMP-independent protein kinases may play a role as glutamate dehydrogenase-inactivating enzymes.

Cyclic AMP-dependent inhibition of glutamate dehydrogenase activity could not be observed in crude extract of bcy1 mutant cells (Fig. 1). To find the difference between wild type and bcy1 enzymes, crude extract of bcy1 cells was applied to a DEAE-Sepahcel column, and glutamate dehydrogenase activity was assayed (Fig. 3). The peak of glutamate dehydro-
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**FIG. 2.** DEAE-Sephacel column chromatography of NAD-dependent glutamate dehydrogenase, glutamate dehydrogenase-inactivating activity, protein kinase, 32P-labeled 100K protein and 5'-p-fluorosulfonyl[14C]benzo[y]adenosine-binding protein in a 20,000 × g supernatant fluid of the wild type strain. A 20,000 × g supernatant fluid (15 ml) of the wild type strain (F10D) containing 12.4 mg of protein/ml was applied to a DEAE-Sephacel column (2.0 × 20 cm), and proteins were eluted using NaCl gradient (0–0.8 M) (broken line) in 4-ml fractions. Each fraction was assayed for NAD-dependent glutamate dehydrogenase activity (X), glutamate dehydrogenase-inactivating activity (O, □), protein kinase activity (A, △), 32P incorporation into 100K protein (V, ▽), and 14C radioactivity of 5'-p-fluorosulfonyl[14C]benzo[y]adenosine bound with 100K protein (□). These assays were performed in the absence (open symbols) and presence (closed symbols) of 10 μM cyclic AMP. All values are expressed per fraction.

Glutamate dehydrogenase activity was found at the same position as that of the wild type enzyme on the chromatogram, and no further inhibition of glutamate dehydrogenase activity was observed by the addition of cyclic AMP and ATP. When aliquots of all fractions obtained were incubated with peak fractions of glutamate dehydrogenase activity in the presence of cyclic AMP and ATP, two troughs of glutamate dehydrogenase-inactivating activity were observed on the chromatogram (Fig. 3). The inactivation of the enzyme at both troughs was cyclic AMP-independent. As shown in Fig. 3, the troughs of glutamate dehydrogenase-inactivating activity coincided exactly with peaks of cyclic AMP-independent protein kinases of bcyl mutant cells. These results suggest that NAD-dependent glutamate dehydrogenase of bcyl cells is inactivated by cyclic AMP-independent protein kinases in the absence of cyclic AMP. One of the cyclic AMP-independent protein kinases which inactivated NAD-dependent glutamate dehydrogenase in bcyl cells might have been derived from the catalytic subunit of wild type cyclic AMP-dependent protein kinase as discussed in our previous paper (3).

**Kinetics of Glutamate Dehydrogenase Inactivation.**—The active fractions of NAD-dependent glutamate dehydrogenase of wild type cells obtained from DEAE-Sephacel chromatography were pooled and concentrated by an ultrafilter, Immersible CX 10. The concentrated fraction was applied to a Sepharose 6B column. A single peak of glutamate dehydrogenase activity was observed on the chromatogram, and NAD-dependent glutamate dehydrogenase was eluted at the position corresponding to the molecular weight of approximately 450,000, judging from the elution position of marker proteins (Fig. 4). Crude extract from wild type cells contained 0.88 unit/mg of protein, while the activity of partially purified NAD-dependent glutamate dehydrogenase preparation through Sepharose 6B gel filtration was 211 units/mg of protein. Therefore, NAD-dependent glutamate dehydrogenase was purified about 240-fold with 82% recovery from the crude extract. Cyclic AMP-dependent protein kinase was prepared from wild type and CYR3-I mutant (AM21-2A) strains by DEAE-Sephacel chromatography. Partially purified NAD-dependent glutamate dehydrogenase and cyclic AMP-dependent protein kinase fractions were preincubated with 0.1 mM ATP and various amounts of cyclic AMP (final volume 100 μl) at 25 and 35 °C for 5 min, and the enzyme activity of the reaction mixture, diluted to 1 ml by buffer T, was measured. By using this method, the concentrations of cyclic AMP and ATP could be reduced to 2% of the original mixture, thereby rendering the effects of cyclic AMP and ATP negligible during the enzyme assays. As shown in Fig. 5, the Kₐ values for cyclic AMP in the inactivation of NAD-dependent glutamate dehydrogenase by cyclic AMP-dependent protein kinase from wild type cells at 25 and 35 °C were 1.0 × 10⁻⁸ and 2.0 × 10⁻⁸ M, respectively. However, when cyclic AMP-dependent protein kinase from CYR3-I mutant cells was used, the Kₐ values for cyclic AMP at 25 and
**FIG. 3.** DEAE-Sephacel column chromatography of NAD-dependent glutamate dehydrogenase, glutamate dehydrogenase-inactivating activity and protein kinase in a 20,000 \( \times g \) supernatant fluid of the \( \text{bcyl} \) mutant strain. A 20,000 \( \times g \) supernatant fluid (15 ml) of the \( \text{bcyl} \) mutant strain (AM27-2B) containing 15.0 mg of protein/ml was applied to a DEAE-Sephacel column. All procedures and symbols are the same as those indicated in Fig. 2.

**FIG. 4.** Gel filtration of partially purified NAD-dependent glutamate dehydrogenase from the wild type strain on Sepharose 6B. The peak fractions of NAD-dependent glutamate dehydrogenase from the wild type strain (FlOD) on the DEAE-Sephacel chromatogram were concentrated to 1.0 ml by an Immersible CX 10 ultrafilter, and applied to a Sepharose 6B column (1.5 x 40 cm) and eluted with buffer T. Aliquots from each fraction (1.0 ml) were assayed for glutamate dehydrogenase activity (x), \(^{32}\)P incorporation into 10K protein without (\( \square \)) and with (\( \bigtriangleup \)) 1.0 \( \mu \)M cAMP, and \(^{14}\)C radioactivity of 5'-p-fluorosulfonyl['~C]benzoyladenosine bound with 10K protein in the absence (\( \square \)) or presence (\( \bigtriangleup \)) of NADH (0.1 mM). V\(_{0}\) indicates void volume, and Fer, Cat, and Ald indicate the elution positions of ferritin (\( M_\text{r} = 450,000 \)), calf pancreatic catalase (\( M_\text{r} = 250,000 \)), and calf muscle aldolase (\( M_\text{r} = 168,000 \)), respectively.

35 \( ^\circ \)C were \( 6.0 \times 10^{-7} \) and \( 8.0 \times 10^{-8} \) \( \text{M} \), respectively. These values were similar with \( K_\text{m} \) values for cAMP in the phosphorylation by cAMP-dependent protein kinase, when histone and trehalase were used as substrate (13, 14). The \( K_\text{m} \) value for ATP in the inactivation of NAD-dependent glutamate dehydrogenase by cAMP-dependent protein kinase from wild type cells in the presence of 10 \( \mu \)M cAMP was \( 10 \times 10^{-4} \) M (Fig. 6). This value was the same as the \( K_\text{m} \) value for ATP of cAMP-dependent protein kinase assayed by using histone as substrate (Fig. 6). These results indicate that NAD-dependent glutamate dehydrogenase may be one of the substrates of cAMP-dependent protein kinase.

Some kinetic parameters of inactivated NAD-dependent glutamate dehydrogenase were determined. As shown in Table I, the phosphorylated form of NAD-dependent glutamate dehydrogenase was determined.
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dehydrogenase indicated a similar $K_m$ value for NAD, but a high $V_{max}$ value for glutarate and a low $V_{max}$, compared with the dephosphorylated form of this enzyme. At low substrate concentrations, the velocity of the reaction is approximately proportional to the ratio $V_{max}/K_m$ as can be seen in Table I, the ratio of dephosphorylated form was about 100-fold higher than that of the phosphorylated form.

Phosphorylation of NAD-dependent Glutamate Dehydrogenase by cAMP-dependent Protein Kinase and Binding of an NADH Analog—Partially purified NAD-dependent glutamate dehydrogenase was incubated with cAMP-dependent protein kinase of the wild type strain obtained by DEAE-Sepharose chromatography (Fig. 2) and [$γ-^{32}P$]ATP in the absence or presence of cAMP, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Only one phosphorylated protein band was observed on an autoradiogram of gels, and the phosphorylation of the protein was dependent on the presence of cAMP (Fig. 7, A and B). The molecular weight of this protein was estimated to be 100,000, and this protein is referred to as the 100K protein. When partially purified NAD-dependent glutamate dehydrogenase was incubated with cAMP-independent protein kinase of the wild type strain obtained by DEAE-Sepharose chromatography (Fig. 2) and [$γ-^{32}P$]ATP in the absence or presence of cAMP, the 100K protein was phosphorylated even in the absence of cAMP (data not shown).

The inactivation of glutamate dehydrogenase activity by protein kinases was proportional to the extent of phosphorylation of NAD-dependent glutamate dehydrogenase, as shown in Fig. 8. The $K_v$ value for cAMP in the phosphorylation of 100K protein was $2 \times 10^{-8}$ M and the same as that of the inactivation of NAD-dependent glutamate dehydrogenase. As shown in Figs. 2 and 4, the 100K protein was phosphorylated in the presence of cAMP-dependent protein kinase and eluted at exactly the same position as that of NAD-dependent glutamate dehydrogenase on the chromatograms.

An analog of NADH, 5'-p-fluorosulfonyl[14C]benzoyladenosine, bound to the 100K protein, and NADH specifically inhibited this binding (Fig. 7, C and D). The 5'-p-fluorosulfonyl[14C]benzoyladenosine-binding protein was co-chromatographed with NAD-dependent glutamate dehydrogenase on the Sepharose 6B column, as shown in Fig. 2. These results suggest that the inactivation of NAD-dependent glutamate dehydrogenase is caused by phosphorylation of this enzyme by protein kinases, and that the 100K protein is one of the subunits of NAD-dependent glutamate dehydrogenase.

Reactivation and Dephosphorylation of Inactive NAD-dependent Glutamate Dehydrogenase by Alkaline Phosphatase—Partially purified NAD-dependent glutamate dehydrogenase sample (0.4 unit) obtained from wild type cells was inactivated by preincubation at 30 °C for various lengths of time in the presence of 0.1 mM [$γ-^{32}P$]ATP, 10 μM cAMP, and cAMP-dependent protein kinase (0.25 unit). The maximal inactivation of NAD-dependent glutamate dehydrogenase and 100K protein phosphorylation was observed after 5 min. After 10 min, the inactivated NAD-dependent glutamate dehydrogenase was then treated with bovine alkaline phosphatase. The extent of reactivation of inactive NAD-dependent glutamate dehydrogenase was proportional to that of dephosphorylation of 100K protein, as shown in Fig. 8. The reactivation of NAD-dependent glutamate dehydrogenase was observed by treatment with crude extract of wild type cells. These results indicate that the phosphorylated NAD-dependent glutamate dehydrogenase was reactivated by dephosphorylation.

**DISCUSSION**

The results presented in this work show that in yeast cells the conversion of NAD-dependent glutamate dehydrogenase from the active enzyme form to an inactive enzyme form is regulated through the phosphorylation of the enzyme by both cAMP-dependent and cAMP-independent protein kinases. It has been reported that in yeast cAMP-dependent protein

**Fig. 7. Phosphorylation and labeling of NAD-dependent glutamate dehydrogenase.** An aliquot of NAD-dependent glutamate dehydrogenase fraction from the wild type strain (F10D) (4.5 units) partially purified through the Sepharose 6B filtration step was incubated with [$γ-^{32}P$]ATP and cAMP-dependent protein kinase partially purified through the DEAE-Sepharose column chromatography step in the absence (A) and presence (B) of 10 μM cAMP. Protein phosphorylation was detected by autoradiography. 100K→ indicates the position of 100K protein phosphorylated by cAMP-dependent protein kinase. Another aliquot of NAD-dependent glutamate dehydrogenase fraction (4.5 units) was labeled with 5'-p-fluorosulfonyl[14C]benzoyladenosine in the absence (C) and presence (D) of 0.1 mM NADH, and the $^{14}C$ incorporation was detected by fluorography.

**Fig. 8. Inactivation and reactivation of NAD-dependent glutamate dehydrogenase by cAMP-dependent protein kinase and alkaline phosphatase.** The NAD-dependent glutamate dehydrogenase fraction from the wild type strain (F10D) partially purified through the Sepharose 6B gel filtration step was incubated with cAMP-dependent protein kinase (0.25 unit) in the presence of 10 μM cAMP and [$γ-^{32}P$]ATP (1,200 cpm/pmol) or cold ATP for various lengths of time. After 10 min, bovine alkaline phosphatase (2,000 units) was added to the aliquots of the reaction mixture, and then the reaction mixture was incubated for various lengths of time. At each time, aliquots were diluted to 500 μl with buffer T, and assayed for glutamate dehydrogenase activity (●) and $^{32}P$ incorporation into 100K protein (●).

**Fig. 6B**

**Fig. 5.** The inactivation of glutamate dehydrogenase activity by protein kinases was proportional to the extent of phosphorylation of NAD-dependent glutamate dehydrogenase, as shown in Fig. 8. The $K_v$ value for cAMP in the phosphorylation of 100K protein was $2 \times 10^{-8}$ M and the same as that of the inactivation of NAD-dependent glutamate dehydrogenase. As shown in Figs. 2 and 4, the 100K protein was phosphorylated in the presence of cAMP-dependent protein kinase and eluted at exactly the same position as that of NAD-dependent glutamate dehydrogenase on the chromatograms.

**Fig. 7.** Phosphorylation and labeling of NAD-dependent glutamate dehydrogenase. An aliquot of NAD-dependent glutamate dehydrogenase fraction from the wild type strain (F10D) (4.5 units) partially purified through the Sepharose 6B filtration step was incubated with [$γ-^{32}P$]ATP and cAMP-dependent protein kinase partially purified through the DEAE-Sepharose column chromatography step in the absence (A) and presence (B) of 10 μM cAMP. Protein phosphorylation was detected by autoradiography. 100K→ indicates the position of 100K protein phosphorylated by cAMP-dependent protein kinase. Another aliquot of NAD-dependent glutamate dehydrogenase fraction (4.5 units) was labeled with 5'-p-fluorosulfonyl[14C]benzoyladenosine in the absence (C) and presence (D) of 0.1 mM NADH, and the $^{14}C$ incorporation was detected by fluorography.
kinase was active in the phosphorylation of muscle glycogen phosphorylase kinase and glycogen synthetase, resulting in the activation and inactivation of the respective enzymes (19). Hemmings (10) indicated that the phosphorylation of NAD-dependent glutamate dehydrogenase from C. utilis was promoted by glutamate starvation and was completely reversible. He found a phosphoprotein phosphatase which dephosphorylated proteins phosphorylated by cAMP-dependent protein kinase and reactivated the phosphorylated form of NAD-dependent glutamate dehydrogenase, but it was not clear what kind of protein kinase could phosphorylate this enzyme in vivo. The present study indicates that NAD-dependent glutamate dehydrogenase is a common substrate of the CAMP-dependent and CAMP-independent protein kinases of S. cerevisiae.

Increased inactivation of partially purified active NAD-dependent glutamate dehydrogenase (Mr = 450,000) was observed to correlate with increased phosphorylation of 100K protein identified by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Peaks of glutamate dehydrogenase activity, 32P incorporation into 100K protein in the presence of protein kinases, and binding of an analog of NADH to 100K protein coincided exactly on the DEAE-Sephacel chromatogram. These results suggest that the 100K protein may be a subunit protein of NAD-dependent glutamate dehydrogenase. It has been reported that the C. utilis NAD-dependent glutamate dehydrogenase is a tetramer (Mr = 460,000) composed of four apparently identical subunits with a molecular weight of 116,000 each (11). A similar molecular form of NAD-dependent glutamate dehydrogenase is predicted in S. cerevisiae. The phosphorylated 100K protein found in the inactive yeast NAD-dependent glutamate dehydrogenase fractions was dephosphorylated and reactivated by treatment with bovine alkaline phosphatase or crude cell extracts which may contain phosphoprotein phosphatase. The results suggest that the interconversion of NAD-dependent glutamate dehydrogenase between active and inactive forms in S. cerevisiae is regulated in vitro by the phosphorylation-dephosphorylation mechanism as indicated by Hemmings in C. utilis (11, 12).

In previous papers, we demonstrated that the boy1 mutation resulted in the deficiency of the cAMP-binding activity and increased production of a CAMP-independent protein kinase (3), and the CYR3 mutation caused structural alteration of the regulatory subunit of CAMP-dependent protein kinase (13). NAD-dependent glutamate dehydrogenase-inactivating activity of wild type cells was found at the same positions as peaks of cAMP-dependent and CAMP-independent protein kinases on the DEAE-Sephacel chromatogram (Fig. 2), but those of the boy1 mutant cells corresponded to the positions of two CAMP-independent protein kinases, one of which was the catalytic subunit of the mutant CAMP-dependent protein kinase (Fig. 2). The CAMP-dependent protein kinase of CYR3 mutant cells showed significantly higher Kᵢ values for activation by cAMP at 35 °C than those of wild type cells when assayed with histone or trehalase (13) as the substrate. Similarly high Kᵢ values for CAMP were observed in the inactivation of active NAD-dependent glutamate dehydrogenase. These results suggest that NAD-dependent glutamate dehydrogenase is one of the in vivo substrates of CAMP-dependent and CAMP-independent protein kinases in yeast cells.

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