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Biochemistry

Tyrosine hydroxylase: A substrate of cyclic AMP-dependent protein kinase*

(catecholamine biosynthesis/protein phosphorylation/tyrosine 3-monooxygenase)

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ABSTRACT Data demonstrating the direct phosphorylation of tyrosine hydroxylase [tyrosine 3-monooxygenase; L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] purified from rat pheochromocytoma by ATP, Mg^{2+}, and cyclic AMP-dependent protein kinase catalytic subunit are presented. The incorporation of phosphate is highly correlated with the activation of the hydroxylase when either the time of preincubation or the amount of protein kinase subunit is varied. The rate of phosphorylation of tyrosine hydroxylase compares favorably with that of H1 histone, a known substrate of protein kinase. Lineweaver–Burk analysis of crude or purified rat pheochromocytoma tyrosine hydroxylase activity, as a function of pterin cofactor concentration, in the absence of ATP, Mg^{2+}, and protein kinase catalytic subunit, yields a curvilinear relationship which can be resolved into two lines, suggesting two enzyme forms with different affinities for pterin cofactor. A fraction of the hydroxylase present in the tumor exists in the activated state, presumably due to the presence of ATP and endogenous protein kinase activity. When the soluble enzyme is activated by cyclic AMP, ATP, Mg^{2+}, and protein kinase, virtually all of the enzyme is converted to the low K_m state. We conclude that tyrosine hydroxylase is a substrate of cyclic AMP-dependent protein kinase *in vitro* and, presumably, *in vivo*.

Tyrosine hydroxylase [tyrosine 3-monooxygenase; L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] (Tyr H'ase) catalyzes the rate-limiting step in catecholamine biosynthesis (1). Several investigators, using Tyr H'ase from various tissues, have reported that the enzyme is activated by incubation with adenosine 3′,5′-cyclic monophosphate (cAMP)-dependent protein kinase, cAMP, ATP, and Mg^{2+} (2–8). The enzyme is also activated as a consequence of nerve stimulation, and it has been proposed that the neurally mediated enhancement of enzyme activity (2) results from activation of adenylate cyclase and increased levels of intraneuronal cAMP (3). Because elevations of intracellular cAMP, activation of cAMP-dependent protein kinase, and protein phosphorylation are associated with electrical stimulation or potassium-induced depolarization of brain slices (9), it has been presumed that either Tyr H'ase or an activator of the enzyme is phosphorylated when levels of cAMP are elevated *in situ*. However, earlier efforts to demonstrate direct phosphorylation of Tyr H'ase by cAMP-dependent protein kinase were not successful (4, 10). More recently, several reports have appeared that suggest that Tyr H'ase may be directly phosphorylated (11–15).

In this communication we report that Tyr H'ase purified from rat pheochromocytoma is phosphorylated in the presence of ATP, Mg^{2+}, and the catalytic subunit of cAMP-dependent protein kinase. The phosphorylation of the enzyme is closely correlated with enhancement of enzyme activity in the presence of subsaturating concentrations of reduced pterin cofactor.

MATERIALS AND METHODS

Materials. ATP, cAMP, L-tyrosine, Tris, and sucrose were purchased from Sigma. Polyacrylamide, bis-polyacrylamide, N,N,N',N'-tetramethylethylenediamine, and ammonium persulfate were purchased from Bio-Rad, and L-[1-^{14}C]tyrosine, [α-^{32}P]ATP, and [γ-^{32}P]ATP (specific activity 10–40 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) were purchased from New England Nuclear. Standards for sodium dodecyl sulfate ($NaDodSO_4$) gel analysis were purchased from Pharmacia; NCS tissue solubilizer was purchased from Amersham; DEAE-cellulose was purchased from Whatman. Hydroxylapatite was purchased from Clarkson Chemical (Williamsport, PA).

Tyr H'ase Assay. Tyr H'ase was assayed by the method of Waymire *et al.* (16) in the crude supernatant and at each step of the purification procedure. In addition to the enzyme preparation, final concentrations in the assay tube were: 200 mM sodium acetate (pH 6.2), 40 mM 2-mercaptoethanol, 1 mM ferrous sulfate, 100 μM L-[1-^{14}C]tyrosine, 250 μg of partially purified aromatic amino acid decarboxylase, and 1 mM 6-methyltetrahydropterin (6-$MePtH_4$) in a total volume of 0.2 ml. Tubes were incubated for 15 min at 37°C (16). One unit of Tyr H'ase activity is defined as that amount of Tyr H'ase necessary to catalyze the conversion of 1 nmol of L-[1-^{14}C]tyrosine to L-[1-^{14}C]dopa in 1 min at 37°C.

One-Step Activation Assay. To assess the activation of both dialyzed crude supernatant and purified Tyr H'ase, we modified the above assay by addition of 40 mM NaF and 6 mM magnesium acetate and the substitution of 0.1 mM ferrous ammonium sulfate for 1 mM ferrous sulfate. The time of the incubation at 37°C was shortened to 10 min. Tyr H'ase was activated by addition of 100 μM cAMP and 0.5 mM ATP to the assay. The source of protein kinase activity in this assay was from both the endogenous protein kinase in the supernatant and the protein kinase in the crude decarboxylase preparation.

Two-Step Activation of Purified Tyr H'ase. In studies in which both enzyme activation and enzyme phosphorylation were examined simultaneously, the enzyme was preincubated either for different durations at 30°C or for 15 min in the presence of different amounts of cAMP-dependent protein kinase catalytic subunit prepared as described below. Purified enzyme, 5.9 μg, was incubated in the presence of 50 mM potassium phosphate, pH 6.5/6 mM magnesium acetate/2 mM dithiothreitol/0.05 mM [γ-^{32}P]ATP (specific activity 2–6 × 10^5

Abbreviations: 6-$MePtH_4$, 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine; $NaDodSO_4$, sodium dodecyl sulfate; Tyr H'ase, tyrosine hydroxylase; cAMP, adenosine 3′,5′-cyclic monophosphate.

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cpm/nmol) in a final volume of 0.1 ml. After the preincubation, 10 μl was removed for assay of Tyr H'ase, and 2 ml of 25% trichloroacetic acid at 4°C was added to terminate the protein kinase reaction. The precipitated protein was collected by Millipore filtration, the filter was washed three times with 2.0 ml of 25% trichloroacetic acid, and radioactivity was measured by liquid scintillation spectrometry.

The 10-μl aliquot was assayed for Tyr H'ase in the presence of 200 mM sodium acetate, pH 6.2/0.5 mM ferrous sulfate/100 μM L-[1-^{14}C]tyrosine/200 μM 6-MePtH$_4$ in a final volume of 0.2 ml. After incubation at 37°C for 5 min, the reaction was terminated by addition of 0.1 ml of 1 M sodium phosphate (pH 8.0), containing 20 mM 3-iodotyrosine and 250 μg of partially purified L-aromatic amino acid decarboxylase, which was prepared from hog kidney cortex (16). The tubes were capped and incubated for an additional 30 min in order to complete the decarboxylation of dopa and trap the $^{14}CO_2$ (16).

Purification of Tyr H'ase. The enzyme was obtained from a transplantable rat pheochromocytoma developed by Warren and Chute (17). The tumors were serially passed by a slight modification of the method of Chalfie and Perlman (18). After removal, the tumors were stored at −80°C with no loss of enzyme activity for up to 3 months. All operations were performed at 4°C unless noted otherwise. Routinely, 15–20 g of the recently thawed tumor tissue was homogenized in 200 ml of 50 mM potassium phosphate, pH 7.0/0.32 M sucrose/7.5 mM 2-mercaptoethanol at full speed for 1 min in a Sorvall OMNI Mixer. After centrifugation at 108,000 × *g* for 60 min, the pH of the supernatant was adjusted to 7.8 with 2 M NaOH.

Tyr H'ase was precipitated with solid ammonium sulfate, between 30% (16.8 g/100 ml) and 42% (24.4 g/100 ml) saturation, and centrifuged and the pellet was suspended in 20 ml of dialysis buffer. The enzyme was then dialyzed overnight against 50 mM potassium phosphate, pH 7.0/7.5 mM 2-mercaptoethanol. The material was then loaded on a 5 × 15 cm DEAE-cellulose column equilibrated with 50 mM potassium phosphate, pH 7.0/7.5 mM mercaptoethanol/1 mM EDTA. The column was washed at a flow rate of 3 ml/min with 300 ml of equilibration buffer containing 40 mM NaCl. The major portion of Tyr H'ase activity subsequently was eluted with 300 ml of 120 mM NaCl dissolved in the equilibration buffer. Five-milliliter fractions were collected and assayed, and the enzyme-containing fractions, which generally were found in elution fractions 20–40, were pooled and adjusted to pH 7.6 with 2 M NaOH. Tyr H'ase was precipitated with 44% (25.7 g/100 ml) ammonium sulfate. The pellet was dissolved in 20 ml of 10 mM potassium phosphate, pH 8.2/10% glycerol (vol/vol)/1 mM dithiothreitol, and the solution was placed on a 3 × 10 cm hydroxylapatite column equilibrated in the same buffer. Tyr H'ase activity was eluted at a flow rate of 1 ml/min, with a linear gradient of 10–200 mM potassium phosphate (pH 8.0), in 500 ml total volume. Three-milliliter fractions were collected and assayed and the active fractions were pooled. The activity eluted between 30 and 60 mM potassium phosphate. The enzyme was concentrated by ammonium sulfate precipitation (50% saturation, 29.8 g/100 ml). The pellet was resuspended in 0.4 ml of dialysis buffer and dialyzed for 2 hr in 100 vol of 50 mM Tris·HCl, pH 8.2/1 mM dithiothreitol. This material was then placed on two 5–20% linear sucrose density gradient tubes made up in 50 mM Tris·HCl, pH 8.2/1 mM dithiothreitol. The tubes were centrifuged for 20 hr at 2°C in a Beckman SW 40 rotor at 40,000 rpm, and 0.3-ml fractions were collected and assayed for Tyr H'ase. Active fractions were again pooled. The results of a typical purification starting with 16 g wet weight of the frozen tumor are presented in Table 1. Active fractions from the sucrose gradient routinely had specific activities between 300 and 505 units/mg of protein. The enzyme had a high degree of purity based on the presence of a single band after NaDodSO$_4$ gel electrophoresis (see Fig. 3*A*). With preparations of specific activity less than 505 units/mg, the amount of enzyme used was estimated, based on the value of 505 units/mg as the specific activity for pure Tyr H'ase.

Table 1. Purification of Tyr H'ase from rat pheochromocytoma

Step	Activity, units	Protein, mg	Specific activity, units/mg
100,000 × *g* supernatant	4760	768	6.2
30–42% $(NH_4)_2SO_4$	3200	133	24
DEAE eluate	1180	13	91
0–44% $(NH_4)_2SO_4$	1890	6	316
Hydroxylapatite eluate	591	2.5	236*
0–50% $(NH_4)_2SO_4$	525	1.2	438
Sucrose density gradient	221	0.6	368*

* The paradoxical fall in the specific activity of the enzyme at these steps in the purification is attributed to the sulfate-induced activation of the enzyme as a consequence of the ammonium sulfate fractionation steps (19).

Protein Kinase. cAMP-dependent protein kinase catalytic subunit was prepared from bovine cardiac muscle by the method of Sudgen *et al.* (20) through the second hydroxylapatite step. The specific activity of the catalytic subunit preparation was 1.0–1.4 μmol of ^{32}P transferred per mg of protein per min at 30°C. A homogeneous preparation of cAMP-dependent protein kinase catalytic subunit, when assayed under these conditions, has been reported to have a specific activity of 1.85 μmol of ^{32}P per mg of protein per min (20).

Hl Histone. The histone was prepared by method I of Johns (21).

Electrophoresis. NaDodSO$_4$ gel electrophoresis was performed by the method of Laemmli (22). Standards used to determine the subunit molecular weight of Tyr H'ase were bovine serum albumin (dimer, M_r 134,000; monomer, M_r 67,000), catalase subunit (M_r 60,000), ovalbumin (M_r 45,000), and lactate dehydrogenase subunit (M_r 36,000).

All protein concentrations were determined by the method of Lowry *et al.* (23), with bovine serum albumin as the standard.

RESULTS

Purified Tyr H'ase from rat pheochromocytoma can be activated under conditions that are optimal for protein phosphorylation when assayed at subsaturating cofactor concentrations. The activated enzyme showed a decreased K_m (from 480 μM to 120 μM) for the pterin cofactor, 6-MePtH$_4$ (Fig. 1 *A* and *B*), with no change in either the K_m for the substrate tyrosine (74 μM) or the V_{max} of the reaction (Fig. 1*C*). Under identical assay conditions, the crude Tyr H'ase was also activated in a similar manner when exposed to phosphorylating conditions. Both crude and purified Tyr H'ase, in the absence of exogenous cAMP, ATP, and protein kinase, exhibited linear kinetics when analyzed by the method of Lineweaver and Burk at different tyrosine concentrations (Fig. 1*C*). However, when a similar analysis was performed in the presence of different cofactor concentrations, a deviation from linear kinetics was obtained (Fig. 1*B*). The observed nonlinear kinetics were transformed to linear kinetics when Tyr H'ase was assayed under conditions that were optimal for protein phosphorylation.

To verify that only cAMP-dependent protein kinase, ATP, and Mg^{2+} are necessary for the activation of Tyr H'ase, we preincubated a purified preparation of cAMP-dependent protein kinase catalytic subunit with purified Tyr H'ase. The

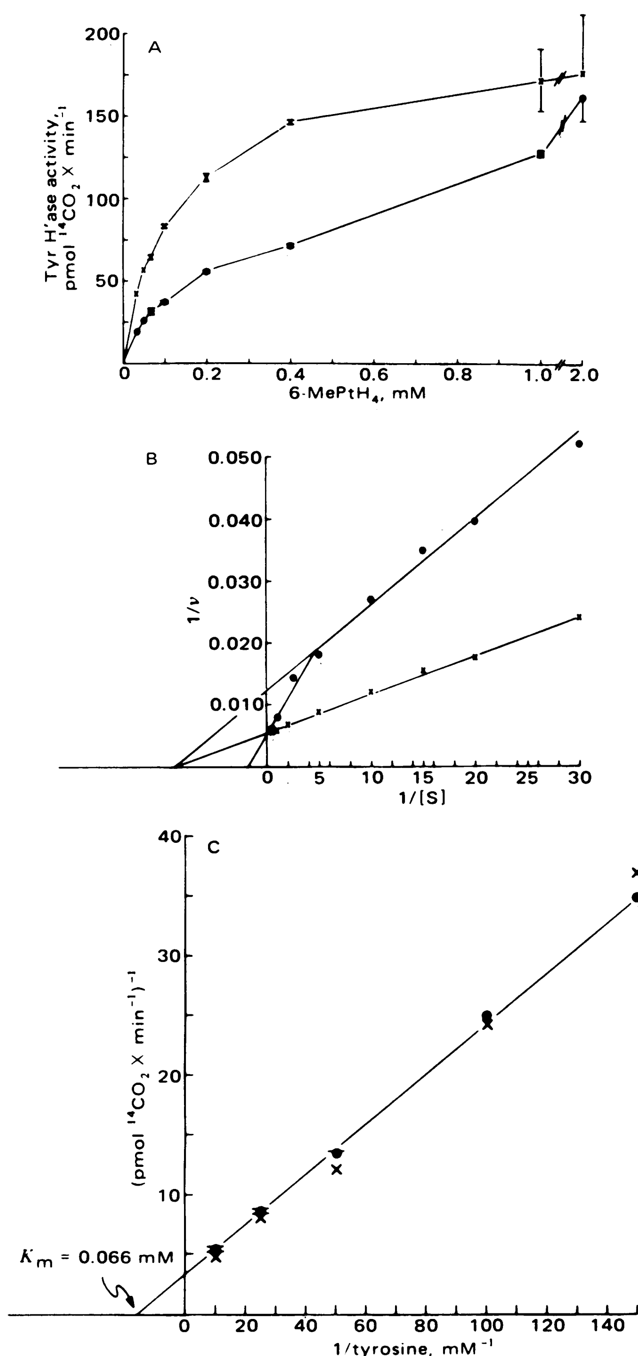

FIG. 1. Activation of purified Tyr H'ase from rat pheochromocytoma. Purified enzyme, 0.65 μg, was assayed by the one-step procedure. ●, Control; X, control plus 0.5 mM ATP and 100 μM cAMP ("activated"). (*A*) Substrate velocity curve for control and activated Tyr H'ase with different concentrations of substrate 6-MePtH$_4$. (*B*) Lineweaver–Burk analysis of data in *A*. (*C*) Lineweaver–Burk analysis of the control and activated Tyr H'ase with different concentrations of tyrosine and 1 mM 6-MePtH$_4$.

activation of Tyr H'ase was completely dependent upon the presence of Mg^{2+} (data not shown), ATP, and catalytic subunit (Fig. 2*A*) and proportional to the amount of catalytic subunit present during the 15-min preincubation at 30°C. In the presence of a fixed amount of catalytic subunit (2 μg), the activation increased in proportion to the duration of the preincubation, up to 15 min (Fig. 2*B*).

The activation of Tyr H'ase was highly correlated with the amount of ^{32}P incorporated into trichloroacetic acid-precipi-

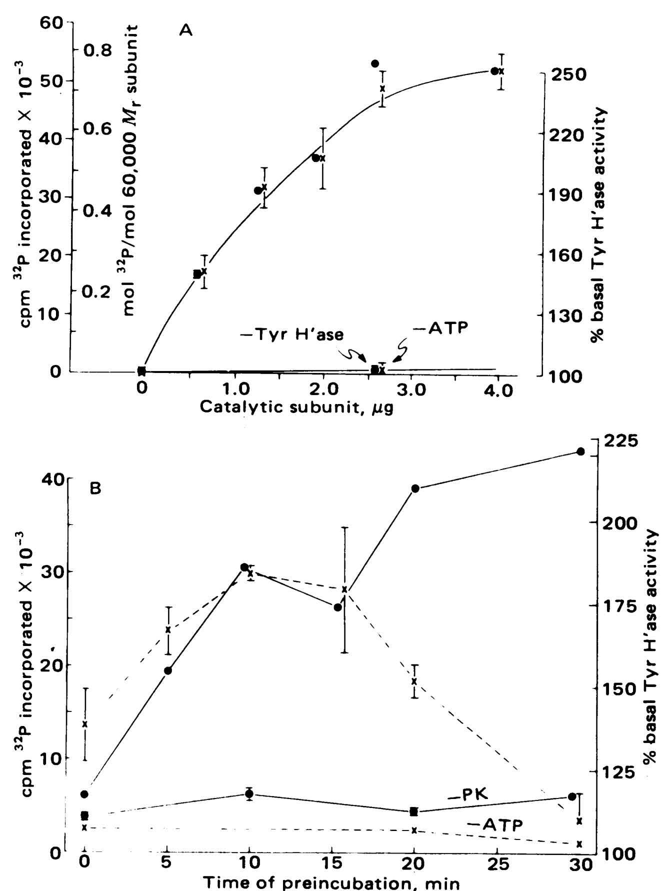

FIG. 2. Phosphorylation and activation of purified Tyr H'ase with highly purified cAMP-dependent protein kinase catalytic subunit. X, Basal Tyr H'ase activity; ●, cpm ^{32}P incorporated (mol ^{32}P/mol subunit). (*A*) Tyr H'ase, 0.07 nmol (5.9 μg; specific activity 360 units/mg), was preincubated with different amounts of catalytic subunit for 15 min at 30°C in 50 mM potassium phosphate, pH 6.5/6 mM magnesium acetate/2 mM dithiothreitol/0.05 mM [γ-^{32}P]ATP. After the preincubation, 10-μl aliquots were removed and assayed for Tyr H'ase activity. The remainder was precipitated with trichloroacetic acid and filtered through a Millipore filter, and radioactivity was measured. The ^{32}P radioactivity was corrected for the amount of Tyr H'ase removed for the enzyme assay. (*B*) Tyr H'ase, 0.07 nmol, was incubated under the same conditions as described in *A* for different times at 30°C. The amount of catalytic subunit present during the incubation was 2 μg. Very little protein phosphorylation was obtained when catalytic subunit of protein kinase was omitted (−PK).

table protein when the preincubation was carried out in the presence of different amounts of catalytic subunit, [γ-^{32}P]ATP, and Mg^{2+}. With a fixed amount of catalytic subunit, the time course of the activation was correlated with ^{32}P incorporation for up to 15 min at 30°C (Fig. 2*B*). After 15 min, phosphate incorporation continued to increase, although Tyr H'ase activity declined toward basal levels. Virtually no incorporation of ^{32}P into protein was observed in the absence of protein kinase catalytic subunit (Fig. 2*B*). In addition, no incorporation of ^{32}P into protein was observed when [α-^{32}P]ATP was substituted for [γ-^{32}P]ATP (data not shown).

Under conditions in which Tyr H'ase was fully activated in the presence of [γ-^{32}P]ATP, approximately 0.7 mol of phosphate was incorporated per mol of 60,000 M_r subunit of Tyr H'ase (Figs. 2*A* and 3*A*). If the preincubation time was limited to 10 min, phosphorylation of other proteins was not observed. When the enzyme preincubation was allowed to proceed for 20 min at 30°C, in addition to the phosphorylation of Tyr H'ase,

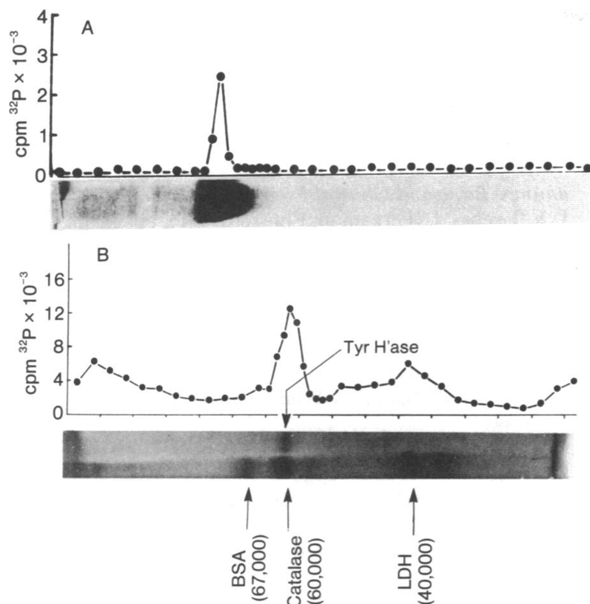

FIG. 3. NaDodSO4 gel electrophoresis of activated Tyr H'ase. (*A*) Tyr H'ase, 10 μg, was preincubated with [γ-^{32}P]ATP, Mg^{2+}, and protein kinase catalytic subunit for 10 min at 30°C under conditions described for the two-step activation assay. After the preincubation, a 10-μl aliquot was removed for assay of Tyr H'ase. The remainder was precipitated with trichloroacetic acid, washed with acetone, and analyzed by NaDodSO4 gel electrophoresis. The gel stained with Coomassie blue represents 20 μg of marker Tyr H'ase run simultaneously in an adjacent well. The larger band width for marker Tyr H'ase vis-à-vis the ^{32}P peak is probably due to the greater loading of the gel with Tyr H'ase protein for the marker well. After electrophoresis, the gel containing the ^{32}P-labeled enzyme was cut into slices and radioactivity was measured by liquid scintillation spectrometry. These results are appropriately positioned (●) beside the protein band in the stained gel. (*B*) Tyr H'ase, 5 μg, incubated for 20 min at 30°C and then analyzed as described in *A*. BSA, bovine serum albumin; LDH, lactate dehydrogenase.

phosphorylation of a protein of M_r approximately 40,000 was apparent (Fig. 3*B*). This peak may represent the phosphorylation of the catalytic subunit of protein kinase after thermal inactivation (24). Phosphorylation of many proteins that are not regarded as physiological substrates of protein kinase has been observed either after chemical modification of the protein (25) or by use of a thermally denatured protein. The reason for the decline of Tyr H'ase activity after 15 min is unclear; the control Tyr H'ase maintained full activity throughout this preincubation. It is possible that the phosphorylated enzyme is less stable than the nonphosphorylated form.

To further evaluate Tyr H'ase as a substrate of cAMP-dependent protein kinase, we compared the relative rates of phosphorylation of Tyr H'ase and H1 histone under conditions described for the preincubation step of the two-step activation assay. The rate of phosphate incorporation into Tyr H'ase was found to proceed at approximately 2.0–2.5 times the rate of H1 histone phosphorylation (Table 2).

DISCUSSION

A fundamental requirement for proof of the hypothesis of direct phosphorylation as a mechanism for enzyme regulation is the demonstration of the simultaneous activation and phosphorylation of the enzyme (26). However, this has not been reported for a highly purified preparation of Tyr H'ase. In addition, data on the stoichiometry of phosphate incorporation per mole of Tyr H'ase subunit and a comparison of the phosphorylation of Tyr H'ase with other substrates of protein kinase have not been presented previously. By use of a highly purified preparation of Tyr H'ase, the relationship between Tyr H'ase activation and direct phosphorylation was examined in this study.

Table 2. Comparison of the relative rates of phosphorylation of H1 histone and Tyr H'ase

Substrate	Incubation at 30°C, min	nmol ^{32}P/ nmol substrate
Histone H1	5	0.10
Tyr H'ase	5	0.20
Histone H1	15	0.24
Tyr H'ase	15	0.59

Protein substrates (0.04–0.07 nmol) were incubated under conditions described for the two-step activation assay in the presence of cAMP-dependent protein kinase catalytic subunit, [γ-^{32}P]ATP, and Mg^{2+}.

Both the dialyzed 100,000 × *g* pheochromocytoma supernatant enzyme and the purified Tyr H'ase exhibit nonlinear kinetics by Lineweaver–Burk analysis when assayed at different 6-MePtH4 concentrations. These "curved-line" kinetics are indicative of either negative cooperativity or the presence of two forms of the enzyme (Fig. 1*B*) (27). Exposure of the enzyme to phosphorylating conditions results in activation of the enzyme, accompanied by a conversion of the Lineweaver–Burk relationship to a linear form. The conversion from curved-line kinetics to straight-line kinetics is compatible with the conversion of Tyr H'ase from a state in which both the less active and more active forms of enzyme, with differing K_m values for pterin cofactor, coexist to a state in which the enzyme is almost exclusively in the more active form. Similar kinetics are observed for Tyr H'ase from guinea pig vas deferens (2) and adrenal medulla (6). By using the equations reported for the determination of the relative amounts of two forms of an enzyme with different substrate affinities but similar maximal velocities (6, 28), we estimate that, in the purified preparation (Fig. 1*B*), 30–40% of the Tyr H'ase exists in the activated form.

If the hypothesis of direct enzyme phosphorylation is correct, then a direct relationship between the amount of phosphate incorporated and the degree of activation of Tyr H'ase should be observed. This correlation is obtained both when the amount of catalytic subunit added (Fig. 2*A*) and when the length of the preincubation under phosphorylating conditions, for up to 15 min at 30°C, are varied (Fig. 2*B*). As indicated in Fig. 2*A*, the calculated stoichiometry approaches 0.7 mol of phosphate per mol of Tyr H'ase subunit as Tyr H'ase approaches full activation. The calculation is based on the specific activity [γ-^{32}P]ATP used and the calculated molar amounts of Tyr H'ase subunit added to the assay tube. The value does not approach 1 mole of phosphate per mole of M_r 60,000 subunit because added enzyme is probably partially phosphorylated, as indicated in the kinetic analysis described above. These results are analogous to those obtained by Milstien *et al.* (29) for phenylalanine hydroxylase. This fact, coupled with the appearance of only a single M_r 60,000 subunit on NaDodSO4 gel analysis that is coincident with the phosphorylated protein (Fig. 3*A*) (30), suggests that the native form of Tyr H'ase is composed of identical subunits all of which can be phosphorylated. These data, as well as the kinetic changes that we have observed, are at variance with those recently reported by Joh *et al.* (13) for rat caudate Tyr H'ase. The latter workers found several enzyme subunits of different molecular weight, only one of which was phosphorylated. They also observed only a change in V_{max} when rat caudate enzyme was incubated in the presence of protein kinase catalytic subunit, ATP, and Mg^{2+}.

The rate of phosphorylation of Tyr H'ase was greater than twice that observed for H1 histone. Comparison of the rates of phosphorylation of H1 histone and Tyr H'ase with analogous data from Cohen *et al.* (31) on various other protein kinase substrates indicates that the rate of Tyr H'ase phosphorylation is faster than that of the α subunit of phosphorylase kinase and slightly less than that of glycogen synthase *in vitro*. This would be expected because all of the proven *in vivo* enzyme substrates of cAMP-dependent protein kinase are phosphorylated at a rate greater than that of H1 histone.

In summary, we have demonstrated the direct phosphorylation of Tyr H'ase by cAMP-dependent protein kinase catalytic subunit. Because nerve stimulation results in activation of the enzyme to a form with kinetic properties similar to those observed for the phosphorylated enzyme (2), it is possible that this is the mechanism by which Tyr H'ase is activated during nerve stimulation. We conclude that Tyr H'ase is a substrate of cAMP-dependent protein kinase *in vitro* and, presumably, *in vivo*.

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