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I am submitting herewith a thesis written by Charles Spielholz entitled "Phosphorylation of Tyrosine Aminotransferase by Cyclic AMP Dependent Protein Kinase." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry.

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PHOSPHORYLATION OF TYROSINE AMINOTRANSFERASE BY
CYCLIC AMP DEPENDENT PROTEIN KINASE

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Presented for the
Master of Science
Degree
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DEDICATION

To my wife Carol, my parents and my sisters, Justine and Sherry.

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ABSTRACT

Tyrosine aminotransferase (E.C. 2.6.1.5, TAT) has been shown to be a phosphoprotein in rat liver and cultured H-35 hepatoma cells (Lee and Nickol, 1974; Wicks and Su, 1978). Stellwagen and Kohli (1981) have shown that phospho-TAT isolated from cultured hepatoma cells may contain at least two tryptic phospho-peptides. They have also shown that TAT can be phosphorylated in vitro by cyclic AMP dependent protein kinase (PK-C).

Undegraded TAT was purified to near homogeneity from rat liver and was confirmed to be a substrate for beef heart PK-C in vitro. A polyclonal antibody, which was found to be specific for TAT, was used to quantitate the amount of phosphate incorporated into the enzyme. Alteration of reaction conditions indicated that phosphate incorporation was maximum with a PK-C to TAT ratio of 7:1 using 200 μ M ATP for 60 minutes at 37°C. The amount of phosphate incorporated into TAT in this work was significantly higher than that obtained by Stellwagen and Kohli (1981): 0.7 moles phosphate/mole TAT subunit versus 0.2 moles phosphate/mole TAT subunit.

Phospho-peptide maps of TAT phosphorylated in vitro by PK-C and TAT obtained from cells incubated with ^{32} P and treated with dexamethasone with and without 8-bromo-cyclic AMP were produced by the method of Cleveland et al. (1977) using α -chymotrypsin, papain and subtilisin. There was no significant difference in phospho-peptide maps of TAT isolated from cells that were treated with 8-bromo-cyclic AMP and cells that were not. Also, the major sites phosphorylated in TAT in vitro by PK-C were not the same as the major sites phosphorylated in vivo. There was, however, some homology between minor sites of phosphorylation in vivo and in vitro. These results

indicate that PK-C is not responsible for most TAT phosphorylation in vivo and that a cyclic AMP independent protein kinase is involved.

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CHAPTER I

INTRODUCTION

Tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, E.C. 2.6.1.5, TAT) is the first enzyme in the pathway of tyrosine catabolism in the liver. The enzyme catalyzes the transfer of the amino group of tyrosine to α -ketoglutarate using pyridoxal 5'-phosphate as a cofactor. The resultant p-hydroxyphenylpyruvate is then metabolized to fumarate and acetoacetate through four enzymatic steps. This pathway is the major route of tyrosine metabolism in mammals (La Du and Gjessing, 1978).

TAT has been under intensive study in several laboratories because its synthesis can be induced by glucocorticoid hormones, cyclic AMP (glucagon) and insulin in rat liver and cultured hepatoma cells by apparently independent mechanisms (Barnett and Wicks, 1971; Wicks, Van Wijk and McKibbin, 1973; Wicks, 1974; Lewis *et al.*, 1982) and because the enzyme undergoes rapid turnover (Johnson and Kenney, 1973). In addition, TAT also exists as a phosphoprotein in rat liver and hepatoma cells (Lee and Nickol, 1974; Wicks and Su, 1978). The physiological significance, the kinases and phosphatases involved and the *in vivo* stoichiometry of this phosphorylation event have not been explored. It is the purpose of this work to begin a study of this phenomenon.

Phosphorylation is an important regulatory mechanism in metabolism. It is considered by some to be ". . . the major general mechanism by which intracellular events in mammalian tissues are controlled by external physiological stimuli." (Cohen, 1982). A large number of proteins are known to be phosphorylated and the functional significance of some of these

phosphorylations is beginning to be understood. Many protein kinases, protein phosphatases, kinase inhibitors, phosphatase inhibitors and regulatory proteins have been described in a wide variety of phosphorylation reactions (Cohen, 1978, 1981, 1982; Ingebritsen and Cohen, 1983; Krebs and Beavo, 1979). Regulatory agents such as cyclic AMP, cyclic GMP, calcium ions and phospholipids also play important roles in protein phosphorylation; these agents mediate the effects of a large variety of hormones, neurotransmitters and other effectors (Greengard, 1978).

In view of the large number of examples already described, the possibility exists that phosphorylation of TAT may be a method of regulating a property of the enzyme. If this is true, phosphorylation would represent a method of regulation in addition to control of the aminotransferase's synthesis. There are other inducible enzymes which are subject to phosphorylation including ATP citrate lyase, acetyl CoA carboxylase, phenylalanine hydroxylase, tyrosine hydroxylase and HMG-CoA reductase (Krebs and Beavo, 1979); therefore TAT is not unique in this respect. Indeed, regulation of TAT might be expected to be quite complex. There is evidence that tyrosine blood levels may be directly related to neuroactive catecholamine levels in the central nervous system (Wurtman et al., 1974) and that a deficiency of TAT can cause tyrosinemia, a disease which is manifested by mental retardation, hepatic and renal malfunction as well as visual impairment (Garibaldi, 1977). Although TAT is a minor liver enzyme, it is a significant enzyme metabolically and its regulation might be expected to be complex. A study of the site, stoichiometry and functional impact of TAT phosphorylation appears to be worthwhile.

Although the phosphorylation of TAT has not been well studied, there is some information about the nature of this phenomenon. A preliminary

observation about the site of phosphorylation in vivo indicates that the phosphate does not seem to be located in the sequence of amino acids which constitute the 3500 dalton C-terminal peptide released by proteolysis (Hargrove et al., 1982).

The kinase responsible for the phosphorylation of TAT in rat hepatoma cells was originally interpreted to be cyclic AMP independent (Wicks and Su, 1978). This conclusion was reached because, although phosphorylation of the aminotransferase was increased by all three inducers of TAT, only one of the inducers (dibutyryl cyclic AMP) was able to activate cyclic AMP dependent protein kinase (ATP:protein phosphotransferase, E.C. 2.7.1.37, cAMP-PK); the other two (insulin and dexamethasone) could not (Wimalasena and Wicks, 1979). Even though the line of hepatoma cells used had little or no cAMP-PK activity in the absence of added cyclic AMP derivatives (Wimalasena et al., 1979), the extent of the increase in TAT phosphorylation during induction by the three inducers was roughly proportional (Wicks and Su, 1978). Therefore, it was plausible to suggest that the kinase responsible for TAT phosphorylation was cyclic AMP independent.

However, another laboratory has recently shown that TAT can be phosphorylated in vitro by the catalytic subunit of cAMP-PK (PK-C) and also by cyclic GMP dependent protein kinase (cGMP-PK) (Stellwagen and Kohli, 1981). Preliminary tryptic peptide analysis by Stellwagen has indicated that TAT isolated from cultured cells may be phosphorylated at two different sites and that PK-C may be able to phosphorylate only one of these sites in vitro. These data raise the possibility that TAT may be phosphorylated by two different kinases in vivo: PK-C and a cyclic AMP independent kinase.

It was the purpose of this work to optimize the phosphorylation of TAT in vitro by PK-C and to compare TAT phosphopeptides generated in vitro to those isolated from cultured cells. These data were then to be used to assess the nature of the kinases involved in the phosphorylation of TAT in vivo.

CHAPTER II

PURIFICATION OF UNDEGRADED TYROSINE AMINOTRANSFERASE

Problems Associated with Purification of Undegraded Tyrosine Aminotransferase

Tyrosine aminotransferase (TAT) presents several problems when used as a model enzyme in the laboratory. First of all, the enzyme constitutes less than 0.005% of total soluble liver protein. Secondly, the native enzyme seems to be very susceptible to proteolysis in vitro by cathepsin T, a protease found in hepatic lysosomes (Gohda and Pitot, 1980 and 1981), and standard protein purification procedures lead to low yields as well as less than optimal specific activities.

Several active forms of TAT are produced during purification which differ by their subunit molecular weight as determined by SDS gel electrophoresis (Hargrove et al., 1980a). These various types are formed by the proteolytic activity of cathepsin T which is released from lysosomes during homogenization (Gohda and Pitot, 1980 and 1981). Native tyrosine aminotransferase consists of two subunits, each with a molecular weight 53,000 and is referred to as type I TAT (Hargrove et al., 1980a). This form of TAT is prepared by taking precautions which inactivate cathepsin T. Two other types of TAT, type II and type III, are produced by cleavage of the native enzyme with cathepsin T during purification. All three forms exhibit full enzymatic activity. Hargrove has shown that type II TAT is a dimer in which one of the subunits has a molecular weight of 49,000 and the subunits of type III TAT are 49,000 each.

In studying phosphorylation of TAT in vitro, it is important that the undegraded form of the enzyme be used since it is this form of TAT that is found in intact rat liver and hepatoma cells (Hargrove et al., 1980a)

Fortunately, the three forms of TAT can be separated by hydroxyl-apatite (Hargrove et al., 1980a) or CM-Sephadex C-50 chromatography (Hargrove et al., 1980b). Additionally, the use of a homogenizing buffer with basic pH seems to limit the proteolytic activity of cathepsin T on TAT until the protease can be inactivated by heating (Gohda and Pitot, 1980). The heat step takes advantage of TAT's heat stability in the presence of excess pyridoxal 5'-phosphate (Hayashi et al., 1967).

Unfortunately, TAT is least stable at basic pH (Aviram and Hershko, 1977) and the heat step must be performed immediately after homogenization at pH 8.2. In addition, some loss of native TAT by conversion to types II and III is unavoidable. Finally, as stated above, since TAT is present in small amounts in rat liver, TAT isolations tend to produce limited amounts of undegraded enzyme.

Finally, highly purified TAT is very susceptible to loss of activity upon storage. No known storage conditions have been found which stabilize TAT activity. Some data indicate that TAT is sensitive to oxidation (Kenney, 1962; Buckley and Milligan, 1978; Dicola and Federici, 1982), however, the actual mode of inactivation of TAT in vitro is not known. Addition of substrates and cofactor (α -ketoglutarate, L-tyrosine and pyridoxal 5' phosphate), with β -mercaptoethanol, dithiothreitol, sucrose and/or glycerol at temperatures ranging from -196°C to 4°C does not appreciably enhance TAT's storage life. Because of this problem, most experiments with TAT must be done shortly after its isolation.

Preparation and Assay of Tyrosine Aminotransferase from Rat Liver

The following protocol has been used to isolate TAT in its native form as rapidly as possible. It is a modification of the method of Hargrove and coworkers (1980a). Materials were obtained as follows: 8-bromo-cyclic AMP was synthesized according to Muneyama et al., (1971). Dexamethasone, L-tryptophan, L-tyrosine, HEPES, EDTA, pyridoxal 5'-phosphate, α -ketoglutarate, β -mercaptoethanol, diethyldithiocarbamic acid and phenylmethyl-sulfonyl fluoride (PMSF) were from Sigma; 3-isobutyl-1-methylxanthine was from Aldrich. Ammonium sulfate, enzyme grade, was purchased from Schwartz/Mann. DEAE Cellulose 53 was obtained from Whatman and CM Sephadex C-50 was from Pharmacia. All other chemicals were the best grade available from Fisher. Dialysis tubing with a molecular weight cutoff of 12,000-14,000 was obtained from Spectrum and was incubated in two changes of hot (90°C) 4 mM EDTA before storing in 50% ethanol at 4°C.

Male Sprague-Dawley rats, about 250 grams each, were fasted overnight and injected intraperitoneally the next morning with 1 mg dexamethasone, 2 mg 8-bromo-cyclic AMP, 1 mg 3-isobutyl-1-methylxanthine and 100 mg L-tryptophan in 2 ml of phosphate-buffered saline pH 7.4 to induce TAT synthesis. After 5 hours, the rats were decapitated, their livers rapidly removed and rinsed briefly in ice cold 0.15M KCl. The livers were then weighed to the nearest 5 grams, diced with scissors into 5 volumes of ice cold homogenizing buffer (100mM HEPES, pH 8.2, 0.10 M KCl, 1.0 mM EDTA, 0.2 mM pyridoxal 5'-phosphate, 5.0 mM α -ketoglutarate, 10 mM β -mercaptoethanol and 0.1 mM PMSF in 0.1% isopropyl alcohol) and homogenized in a Waring blender at full speed with two 20 second bursts at room temperature. The homogenate was then heated to 65°C for 5 minutes with constant stirring in a 75°C water

bath. The homogenate was cooled in an ice bath for 20 minutes and centrifuged for 30 minutes at 16,000 x g.

Solid ammonium sulfate was added to the supernatant to 45% saturation while neutral pH was maintained by addition of HCl. This suspension was centrifuged at 16,000 x g for 30 minutes, the precipitate discarded and ammonium sulfate was again added until 70% saturation was reached. This suspension was again centrifuged at 16,000 x g for 30 minutes and the resulting precipitate was dissolved in a small volume of ice cold DEAE buffer (25 mM potassium phosphate, pH 7.4, 0.1 M KCl, 1.0 mM EDTA, 0.2 mM pyridoxal 5'-phosphate, 5.0 mM α -ketoglutarate and 10 mM β -mercaptoethanol) with the aid of a glass dounce homogenizer. The preparation was dialyzed overnight at 4°C against two changes of 4 liters of DEAE buffer. Unless otherwise specified, all the following procedures were carried out at 4°C in a cold room.

The dialyzed enzyme was loaded, at 50 ml per hour, onto a DEAE cellulose 53 column (2.5 cm by 22.5 cm) that had been equilibrated in DEAE buffer. The column was washed with 100 ml of DEAE buffer and eluted with a linear gradient of 500 ml of DEAE buffer and 500 ml of DEAE buffer containing 0.5M KCl at a flow rate of 50 ml per hour. Collected fractions (about 8 ml each) were assayed for TAT activity (described below) and active fractions were pooled (Figure 2.1).

Pooled fractions were dialyzed overnight against two 4 liter changes of CM buffer (25 mM sodium phosphate, pH 6.5, 1.0 mM EDTA, 2.5 mM α -ketoglutarate and 10mM β -mercaptoethanol) and loaded onto a freshly prepared CM Sephadex C-50 column (2.5 cm by 21.0 cm) at 30 ml per hour. The column was eluted with an 800 ml linear gradient of KCl from 0 to 0.4 M in CM buffer a rate of 30 ml per hour. The fractions (about 6.5 ml each)

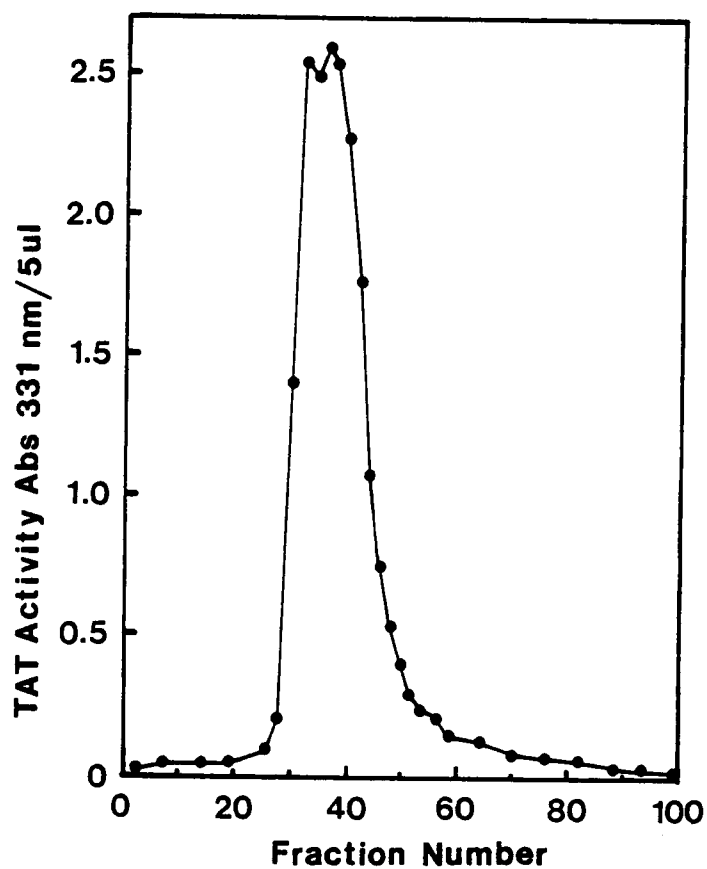


Figure 2.1. DEAE cellulose chromatography of tyrosine aminotransferase. 1.13×10^6 units of TAT was loaded onto and eluted from the column as described in the text. 5 μ l of each 8ml fraction was assayed for TAT activity which is expressed as absorbance at 331nm. Fractions 28 to 50 were pooled from this column. There was virtually no TAT activity in the flow through.

were assayed for TAT activity. At least two overlapping peaks of TAT activity were present in all enzymatic preparations (Figure 2.2). The first peak of TAT activity eluted from the column represents the undegraded form of TAT (type I) as shown by SDS gel electrophoresis. The second peak represents type II TAT. (Sometimes a small, third peak of TAT activity representing type III TAT was seen which eluted after the type II peak.)

The fractions identified as containing exclusively type I TAT were pooled and concentrated 12 to 13 fold with an Amicon PM-10 filter membrane under 60 psi of nitrogen. The concentrated enzyme was dialyzed 4 to 5 hours against 25mM potassium phosphate, pH 6.8, 0.2mM pyridoxal 5'-phosphate, 2.5mM α -ketoglutarate and 10mM β -mercaptoethanol before being stored in a plastic tube at 4°C.

TAT activity was measured by a modification of the method of Diamondstone (1966) as described by Wicks (1969). A 2.2 ml reaction mixture containing 2.4 mM L-tyrosine, 40 mM potassium phosphate, pH 7.6, 297 mM pyridoxal 5'-phosphate, 2 mM diethyldithiocarbamic acid and TAT was warmed to 37°C. The reaction was initiated by addition of 0.3 ml of 100mM α -ketoglutarate, pH 7.6, and incubation was carried out for 20 minutes at 37°C. The reaction was stopped by addition of 0.2 ml 10 N NaOH with vigorous vortexing. The mixture was then incubated for 30 minutes at room temperature. The NaOH catalyzes the conversion of p-hydroxyphenylpyruvate to p-hydroxybenzaldehyde (Diamondstone, 1966). The optical density of p-hydroxybenzaldehyde is determined spectrophotometrically at 331nm. Units of TAT activity are determined using an extinction coefficient of 10.77 (Wicks, 1969). One unit of TAT activity is equal to the production of 1 μ g of p-hydroxyphenylpyruvate in 10 minutes at 37°C (Kenney, 1959). Protein was

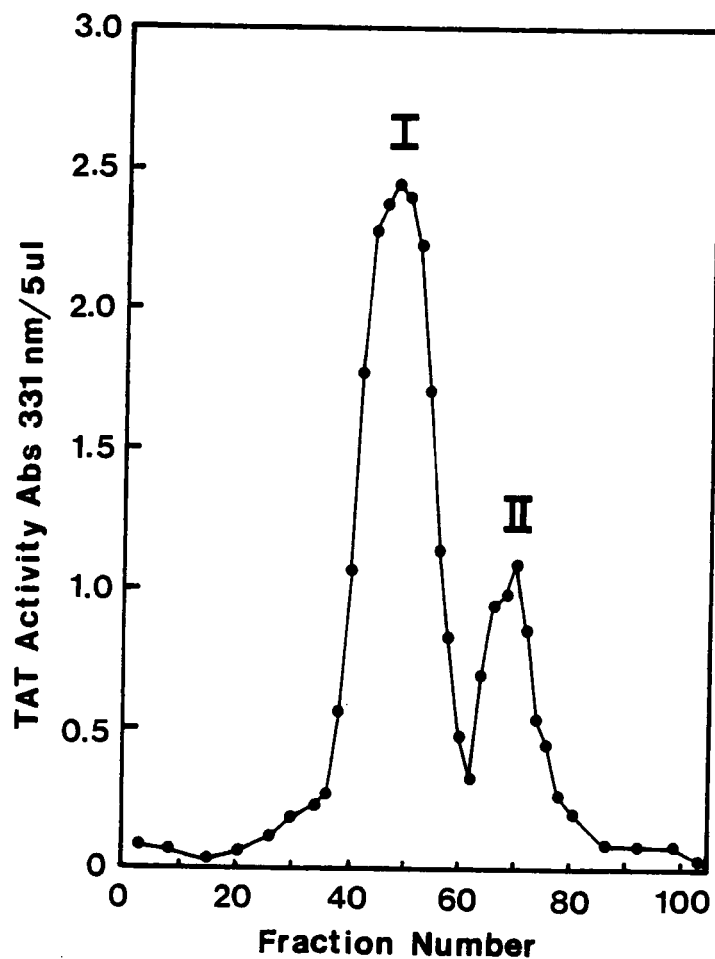


Figure 2.2. CM Sephadex C-50 chromatography of tyrosine aminotransferase. 5.4×10^5 units of TAT was loaded onto and eluted from the column as described in the text. 5 μ l of each 6.6ml fraction was assayed for TAT activity which is expressed as absorbance at 331nm. Fractions 38-58 were identified as type I TAT and pooled. There was 1.15×10^5 units of TAT activity in the flow through of this column.

determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Protein purity was determined by electrophoresis of 10-20 μ g of protein on 1 mm thick 8.5% sodium dodecyl sulfate polyacrylamide gels and staining with Coomassie blue. The composition of the polyacrylamide gels is described in detail in Materials and Methods.

Conclusions

Figure 2.3 shows the SDS-polyacrylamide gel electrophoresis pattern of the three forms of TAT isolated under conditions where some cross contamination of the three forms took place. At this time separation of the three forms on CM-Sephadex C-50 had not yet been perfected. Type III TAT was present in large amounts because homogenization was carried out at pH 7.8 instead of 8.2. In addition to these problems, the overall purity of TAT in these preparations, as judged by electrophoresis, was only about 30%. Figure 2.4 shows a Coomassie blue stained gel of TAT purified under conditions in which no precautions to prevent proteolysis were taken (i.e. homogenization at pH 7.6 in potassium phosphate buffer). The TAT in this preparation has a subunit molecular weight of 47,000 and still retained enzymatic activity. The current isolation procedure, as described above, produces highly purified type I TAT without contamination from type II. Type III is no longer observed when this isolation procedure is used.

Table 2.1 presents a purification summary of a preparation of type I TAT used for many of the experiments to be described in this thesis. As shown in figure 2.5, TAT resulting from this purification was approximately 50% pure as judged by electrophoresis and exhibited a subunit molecular weight of 53,000. Other preparations of TAT using this procedure have produced virtually homogenous enzyme as shown in figure 2.6.

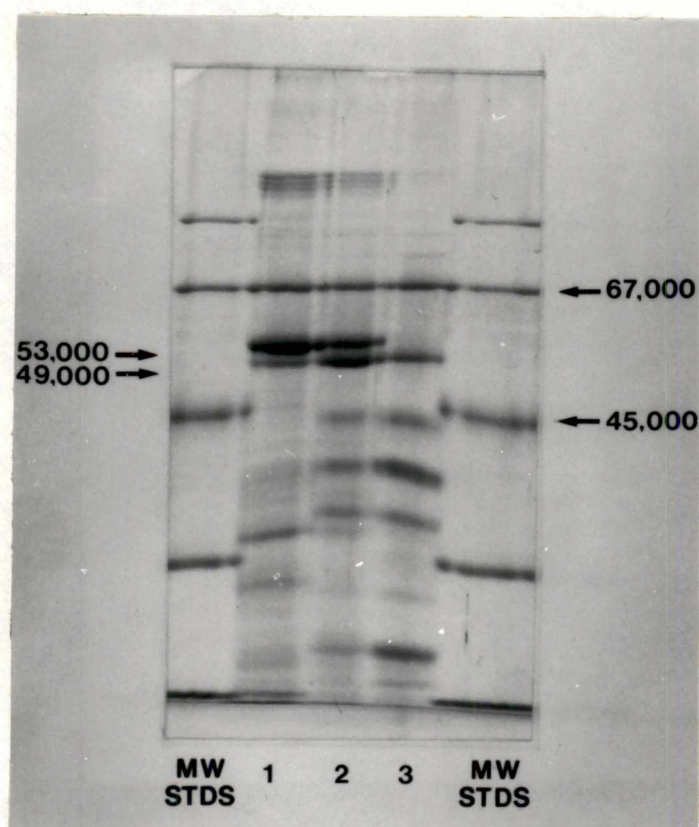


Figure 2.3 Coomassie blue-stained SDS-polyacrylamide gel (8.5%) of the three forms of TAT from CM-Sephadex C-50 eluates. Type I, the undegraded form, has a subunit molecular weight of 53,000 (lane 1). Type II TAT (lane 2) has two different subunits with molecular weights of 53,000 and 49,000. Type III TAT (lane 3) has a subunit molecular weight of 49,000. Each of the three forms in this preparation are cross contaminated and are only 30% pure. The standards and their molecular weights are phosphorylase b (92,500), bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen (25,000) and myoglobin (17,500).

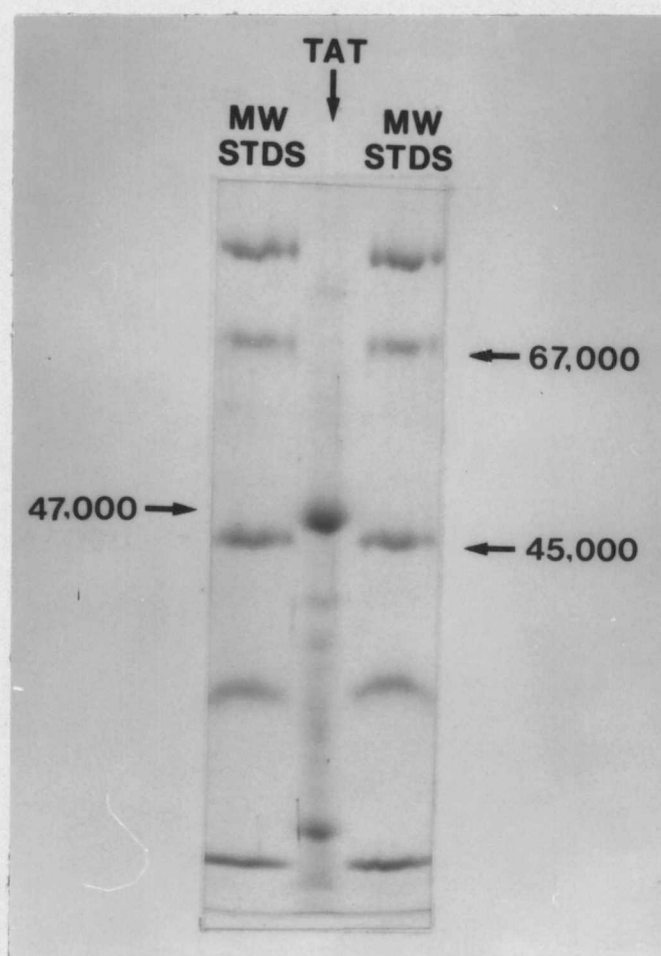


Figure 2.4. Coomassie blue-stained SDS-polyacrylamide gel (8.5%) of TAT purified without precautions to prevent proteolysis. This preparation of TAT has a subunit molecular weight of 47,000 instead of 53,000. The standards are the same as those used in figure 2.3.

Table 2.1

Summary Chart of a TAT Purification Procedure from 115 g Induced Rat Liver^a

Procedure	Volume ml	Total Units	Protein mg	Specific Activity	Yield ^b %	Purifi- ^c cation
Homogenization	870	4.36x10 ⁶	24360	180	100	-
Heat Step	580	2.14x10 ⁶	5104	420	49	2.3X
Ammonium Sulfate Precipitation (45-70%)	94	1.13x10 ⁶	705	1,600	53	3.8X
DEAE-53 Chromatography	191	5.4x10 ⁵	40	13,500	48	8.4X
CM Sephadex C-50 Chromatography	152	2.9x10 ⁵	1.77	164,000	53	12.1X
Concentration under Nitrogen	12	2.6x10 ⁵	1.40	186,000	90	1.1X

^aAs shown in figure 2.5, this preparation of TAT was about 50% pure and had a subunit molecular weight of 53,000.

^bOverall yield was 6%.

^cOverall purification was 1050 fold.

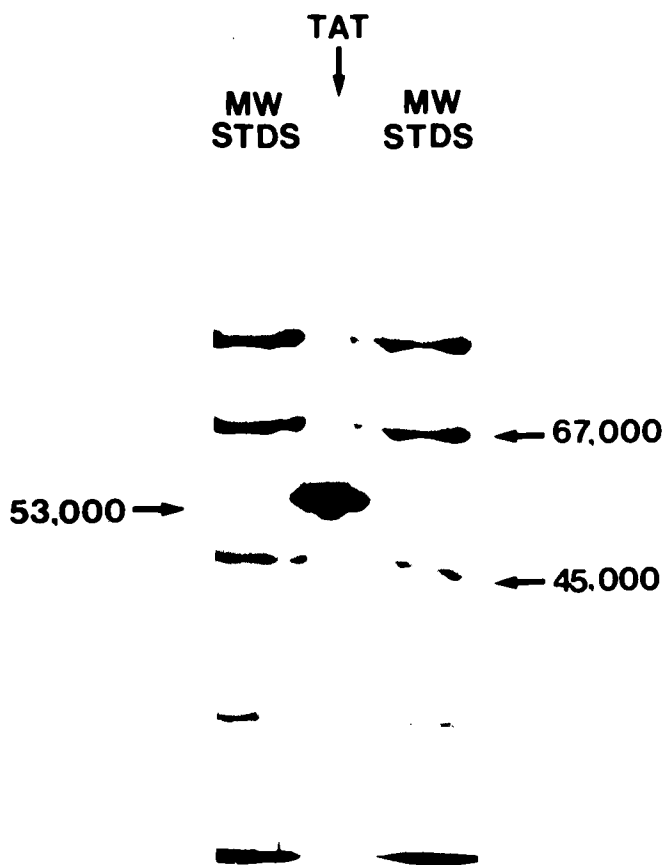


Figure 2.5. Electrophoresis of 12 μ g of the purified tyrosine aminotransferase preparation from table 4.1. The 8.5% polyacrylamide gel was 1mm thick and stained with Coomassie blue. The standards and their molecular weights are phosphorylase b (92,500), bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen (25,000) and myoglobin (17,500). This preparation was estimated to be 50% pure, and had a specific activity of 186,000 units/mg.

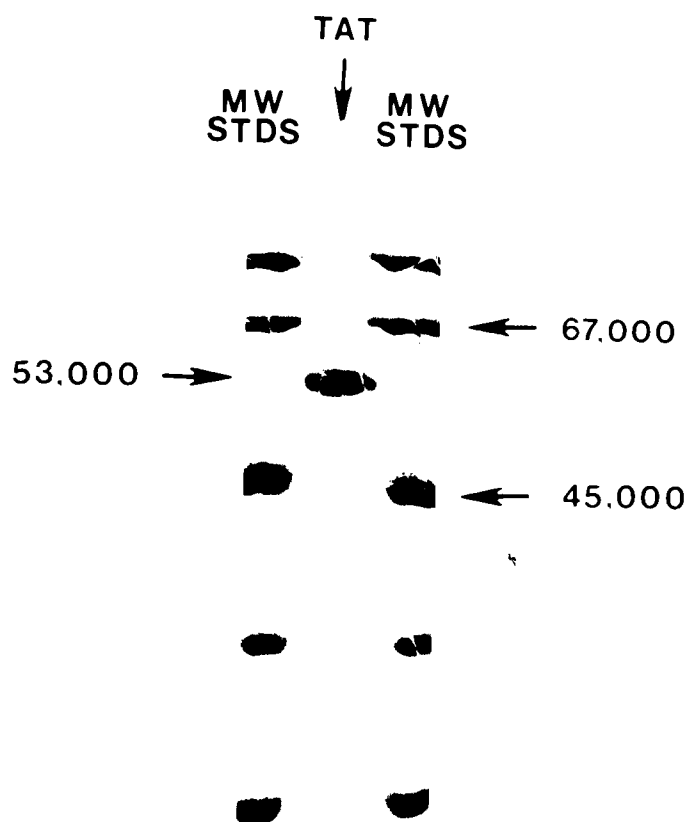


Figure 2.6. Electrophoresis of 12 μ g of near homogenous tyrosine aminotransferase. In this preparation, the enzyme was estimated to be 90% pure and had a specific activity of 184,000 units/mg. See legend of figure 2.5 for gel criteria.

Several points concerning the purification presented in table 2.1 deserve comment. The yield represented in the 45 to 70% ammonium sulfate precipitation is not indicative of a typical preparation. Yields of TAT activity for this step are usually over 95% of the previous step. The reason for the low yield at this step in this particular preparation is not known. The heat step consistently produced a low yield of TAT. This was probably due to heating the enzyme for a long time period at a pH which is not ideal for TAT stability. However, this step is necessary in order to prevent subsequent proteolysis of TAT by Cathepsin T.

The CM-Sephadex C-50 column was useful for separating native TAT from other forms of TAT as shown in figure 2.2. A major difficulty with this column, however, was that about 20% of TAT activity loaded onto the column does not bind and was found in the flow through. Additionally, as seen in Figure 2.2, the peak representing type II TAT overlapped a little with the type I peak. Those fractions containing both type I and type II TAT were not useful for in vitro experiments since type II TAT is not representative of the undegraded form found in intact liver cells. Both the flow through and fractions containing type II TAT were usually discarded.

This method of isolation produced highly purified type I TAT that was used for in vitro experiments. The entire procedure took approximately four days from the time rats were induced for TAT activity. This short preparation time helped to alleviate the difficulties encountered with the low yields associated with the procedure and the short storage life of the enzyme.

CHAPTER III

MATERIALS AND METHODS

Materials

Dexamethasone, α -ketoglutarate, pyridoxal 5'-phosphate, sucrose, triton X-100, sodium dodecyl sulfate (SDS), Tris, acrylamide, bis-acrylamide, ammonium persulfate, β -mercaptoethanol, EDTA, MES, bromophenol blue, Coomassie brilliant blue R and glycine were purchased from Sigma. Dulbecco's modified Eagle medium and BME 100X concentrated vitamin solution were obtained from Gibco. Calf serum and fetal calf serum were bought from Flow Laboratories. (^{35}S)-methionine, carrier free (^{32}P) inorganic phosphate (in water) and Omnifluor were from New England Nuclear. Sodium deoxycholate was obtained from Calbiochem. Nonidet P40 was from Bethesda Research Laboratories; TEMED was purchased from Bio Rad and all other chemicals were the best grade available from Fisher Scientific. Phosphate-buffered saline is 25 mM potassium phosphate, pH 7.6, in 0.9% sodium chloride.

Purification and Assay of PK-C

The catalytic subunit of cAMP dependent protein kinase (PK-C) was purified by Dr. Doris Schlichter and Mr. Keith Carr from beef heart by a modification (Carr, 1984) of the procedures of Bechtel et al. (1977) and Corbin et al. (1978). The PK-C used in these experiments ranged from 50-70% pure to homogenous. Purity was determined by running 20 μg of protein on 8.5% polyacrylamide gels and staining with Coomassie blue as described below. PK-C was assayed by the procedure of McPherson et al. (1979). One unit of PK-C will transfer 1 μmole of phosphate from ATP to histone 2A in one minute. Protein concentration was determined by the method of Lowry et al. (1951) using

bovine serum albumin fraction V (Sigma) as a standard. Molar concentration of PK-C was then estimated using a molecular weight of 41,000.

γ -(^{32}P)-ATP Synthesis

γ -(^{32}P)-ATP was synthesized and purified according to the method of Johnson and Walseth (1979). Unless otherwise specified, all materials for this synthesis were purchased from sources recommended by these authors.

The synthesis uses several steps in the glycolytic pathway. Briefly summarized: L-glycerol-3-phosphate is converted to dihydroxyacetone phosphate by the action of glycerolphosphate dehydrogenase using NAD^+ as the electron acceptor. Triosephosphate isomerase then isomerizes the dihydroxyacetone phosphate to D-glyceraldehyde-3-phosphate. This compound was then phosphorylated with inorganic (^{32}P) through an oxidation reaction catalyzed by glyceraldehyde phosphate dehydrogenase using NAD^+ as an electron acceptor. The resulting 1,3 bisphospho-D-glycerate contained (^{32}P) at the 3 position. The (^{32}P) from this labeled compound was then transferred to ADP through a substrate level phosphorylation catalyzed by phosphoglycerate kinase. During the 20-30 minute reaction, NAD^+ was regenerated from NADH by lactate dehydrogenase using sodium pyruvate as a substrate.

When the reaction was 90-100% complete (as determined by the Norit test; ATP adheres to charcoal, P_i does not), the reaction mixture was loaded onto a Bio Rad AG1-X8(Cl^-) polypropylene Econo Column. The column was washed with water and 30mM HCl and ATP was eluted with 250mM HCl. One ml fractions were collected and fractions containing (^{32}P) were pooled. The pH of the pooled fractions was adjusted to approximately 6.0-6.5 using sodium hydroxide and pH paper.

Purity of the γ -(^{32}P)-ATP was checked by thin layer chromatography (TLC). A known volume of γ -(^{32}P)-ATP containing pure carrier ATP was run on a polyethylene imine cellulose plate (Baker) using a mobile phase of 2M sodium formate followed by 4M sodium formate. This method separates ATP from ADP, AMP and Pi. The spot corresponding to ATP on the PEI cellulose was located using UV light and autoradiography. This spot was cut out and counted and the radioactivity in it was compared to that in a sample of ATP which was not subjected to TLC. The purity of γ -(^{32}P)-ATP preparations used in these experiments was always above 95%. An autoradiograph of a TLC separation of an ATP preparation is shown in Figure 3.1.

In Vitro Phosphorylation of TAT

TAT was phosphorylated in vitro by PK-C using γ -(^{32}P)-ATP as a phosphate donor. The general reaction mixture consisted of 25mM MES buffer, pH 6.8, 5mM magnesium chloride, 4mM β -mercaptoethanol, 200 μM γ -(^{32}P)-ATP (500-2000 cpm/pM), 5-25pM TAT and 5-300pM PK-C (in 0.3M potassium phosphate, pH 6.8, 1mM EDTA, 15mM β -mercaptoethanol). Final reaction volumes varied from 40 to 140 μL . PK-C and TAT each accounted for 25% of the final reaction volume. These conditions are similar to those used to assay PK-C activity (McPherson et al., 1979).

Reactions were always initiated by the addition of ATP, usually carried out for 30 to 60 minutes at 30°C and stopped by the addition of EDTA, pH 7.0, to a final concentration of 50mM. Reaction mixtures were then immunoprecipitated and run on 8.5% polyacrylamide electrophoresis slab gels as described below.

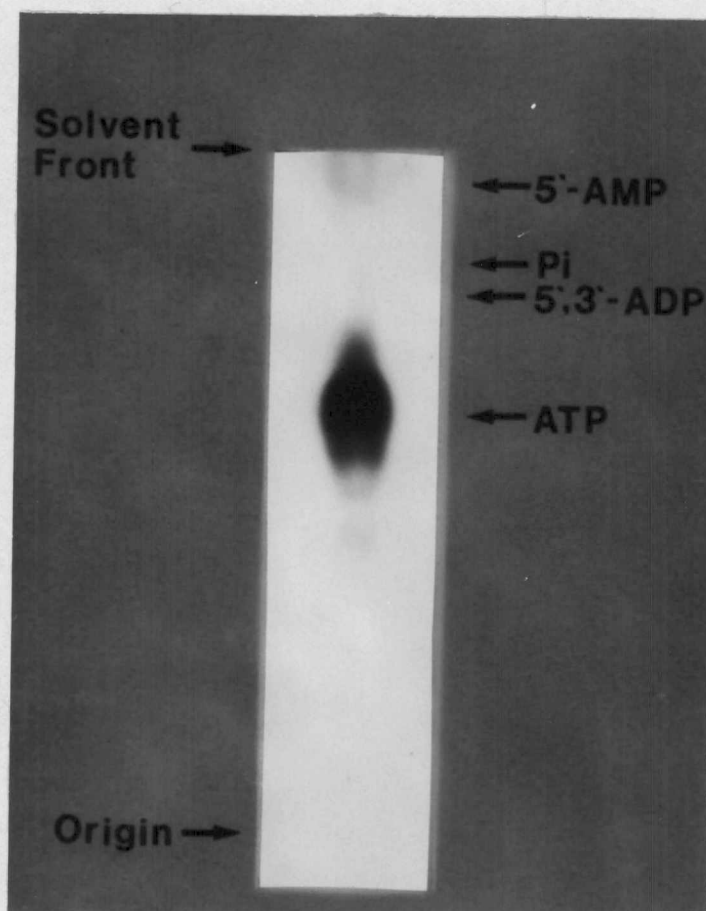


Figure 3.1 Autoradiogram demonstrating the purity of γ -(^{32}P)-ATP as determined by TLC on PEI cellulose. A sample of γ -(^{32}P)-ATP was separated from ADP, AMP and Pi by the method described in the text.

In Vivo Phosphorylation and (^{35}S)-Methionine Labeling of TAT

The cells used in these experiments were a clone (KRC-7) of the Reuber H35 (H4-II-E-C3) cell line (Wicks and Su, 1978). They were grown in plastic cell culture plates 60 mm in diameter with 3 ml of Dulbecco's modified Eagle medium supplemented with 5% calf serum and 5% fetal calf serum (Wicks and Su, 1978). The cells were incubated at 37°C in a humidified CO₂ incubator. When the cells were 85 to 100% confluent, they were incubated overnight in serum-free Dulbecco's modified Eagle medium. The next morning, TAT synthesis was induced in the cells by treating them with either 1 μM dexamethasone for 5 hours or 2 mM 8-bromo-cyclic AMP for 3 hours in serum-free medium (Wicks and Su, 1978). In some experiments, the dexamethasone induction period was overnight.

Incorporation of (^{35}S)-methionine into TAT was accomplished by incubating H35 cells in 3 ml of medium containing $\frac{1}{4}$ the usual amount of L-methionine (7.5mg/ml) along with 100 μCi of (^{35}S)-methionine for 4 hours. Incorporation of ($^{32}\text{P}_i$) into TAT was achieved by incubating H35 cells in 3 ml of medium low in phosphate (1 μM) containing 2mCi of ($^{32}\text{P}_i$) for 2 hours. All labeling was done in the presence of the appropriate inducers as above. Media low in phosphate and methionine was prepared in the laboratory according to the protocol for Dulbecco's Modified Eagle's Medium given in the 1982 Gibco catalogue with the appropriate changes in methionine and phosphate concentrations. All materials for the media were from Sigma except the vitamin solution, which was purchased from Gibco.

At the end of the labeling period, the medium was removed and the cells were washed twice with ice cold 0.9% sodium chloride. The cells were lysed with 1ml of lysis buffer (50 mM potassium phosphate, pH 7.4, 0.12 M potassium

chloride, 5 mM magnesium acetate, 5 mM α -ketoglutarate, 110 μ M pyridoxal 5'-phosphate, 1mM ATP, 0.1% Nonidet P40) and centrifuged in a Brinkman microfuge model 3200 for 10 minutes at 4°C. Samples of this lysate were then used for immunoprecipitation as described below and for TAT activity and protein determinations as described above.

Method of Immunoprecipitation

Immunoprecipitation of labeled tyrosine aminotransferase was carried out according to the procedure of Wicks and Su (1978). Approximately 3500 units of tyrosine aminotransferase antiserum were added to a sample of cell lysate or an incubation mix in a 1.5 ml Eppendorf microfuge tube and were allowed to incubate for 15 minutes at room temperature. Crude unlabeled carrier TAT (2000-4000 units per mg) was then added to a final activity of 3500 units per microfuge tube and the samples were then incubated for 30 minutes at 37°C and 75 to 90 minutes at 4°C.

Each sample was then layered onto a discontinuous sucrose density gradient prepared in a second microfuge tube. The gradient consisted of 0.50 ml of 1.0M sucrose and 0.25 ml of 0.5M sucrose each containing 1% sodium deoxycholate, 1% triton X-100 and 1% sodium dodecyl sulfate (Wicks and Su, 1978). The gradient was centrifuged in a microfuge for 15 minutes at 4°C and the gradient solution was removed with a drawn out Pasteur pipette. The protein pellet was washed four times with a solution consisting of 150mM potassium chloride, 1% Triton X-100 and 1% sodium deoxycholate (pH 7.8).

The pellet was then dissolved in 40 μ l of 10% SDS by incubating it for 2 hours at 60°C. Ten μ l of 5x Stop (0.06M Tris HCl, pH 7.4, 5% SDS, 1.47mM β -mercaptoethanol, 1.5M sucrose, 0.001M EDTA and 0.20 mg/ml bromophenol

blue) was then added and the samples were placed in a boiling water bath for 1 minute. The samples were then subjected to electrophoresis on 8.5% polyacrylamide gels as described below.

Polyacrylamide Gel Electrophoresis (PAGE)

The method of electrophoresis was Maizel's (1971) modification of the procedure described by Laemmli (1970). Running gels consisted of 8.5% polyacrylamide, 0.224% bis-acrylamide, 0.372M Tris base, pH 8.9, 0.05% SDS and 0.250M sucrose and were polymerized by addition of ammonium persulfate to 0.05% and TEMED to 0.1%. The stacking gels were 3% polyacrylamide, 0.08% bis-acrylamide, 0.064M Tris base, pH 6.7 and 0.1% SDS and were polymerized by the addition of ammonium persulfate to 0.25% and TEMED to 0.05%.

Gels were cast in two different modes: slab gels and tube gels. Running gels of slab configurations measured 15 by 15cm. Stacking gels were 15cm wide and 4cm high with wells 2cm deep. Unless otherwise noted, all slab gels were 1mm thick. Tube gels had a diameter of 0.5cm with 9cm long running gels and 1cm long stacking gels.

Slab gels were usually run overnight at 50-80 volts. The gels were removed for staining when the dye front was approximately 0.5cm from the bottom of the running gel. The voltage was increased to 180 volts for the last 6 to 10cm of dye front migration in order to produce sharp bands. Tube gels were run at a continuous 180 volts until dye fronts reached the bottom of the gels. The running buffer for electrophoresis was 0.05M Tris base, pH 8.6-8.8, 0.38M glycine and 0.1% SDS.

Slab gels were stained for at least $\frac{1}{2}$ hour with 0.1% Coomassie brilliant blue R, in 10% acetic acid and 50% methanol, then destained for 1 to 2 hours

with 2 changes of a solution of 10% acetic acid and 10% methanol. Slab gels were then dried under heat and vacuum for 2 hours with white blotting paper as a backing. Tube gels were stained and destained in the same solutions used for slab gels except staining was done for 36 hours and destaining for 4 to 6 days with 2 changes of destain each day. All staining and destaining was done with gentle shaking.

Molecular Weight Determinations from SDS-PAGE

Molecular weight determinations were done using protein standards. The standards were phosphorylase A (92,500), bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen (25,000) and myoglobin (17,000) and were obtained from Sigma.

Autoradiography and Quantitation of Radioactivity in Gels

Labeled proteins bands in slab gels were located by autoradiography for various times using Kodak X-Omat RP X-ray film. In some cases, autoradiography was done using an enhancing screen (Dupont Cronex Lightening-Plus ZE) at -70°C .

Radioactivity due to (^{32}P) was always quantitated in slab gels. The appropriate bands were cut out of dried gels and placed, Coomassie blue side up in scintillation fluid consisting of 0.5% Omnifluor in 37.5% ethylene glycol monomethyl ether and 62.5% toluene. A Beckman LS-7500 liquid scintillation counter set for discrimination of (^{32}P) was used to count samples. This method has been shown to be an efficient method of counting (^{32}P) (Huttner et al., 1981).

In some instances, stoichiometry of in vitro phosphorylated TAT was based on the specific activity of the γ -(^{32}P)-ATP used and a subunit molecular

weight of 53,000 for TAT. TAT concentration was calculated on the basis of protein determinations by the method of Lowry et al. (1951) using bovine serum albumin as a standard and purity estimated by SDS-PAGE. Although this method provides only a rough estimate (since the aromatic amino acid content of bovine serum albumin and TAT are different), it is the same procedure that was used by Stellwagen and Kohli (1981) and will only be used to compare the results of this work to those of Stellwagen and Kohli (1981).

Quantitation of (^{35}S)-methionine-labeled proteins was done in tube gels. After staining, the gels were then frozen in a dry ice-ethanol bath and sliced into 2mm sections with a razor set. Each gel slice was dissolved in 0.25 ml 30% hydrogen peroxide at 37°C for 14 hours and counted in 5 ml of 0.5% Omnifluor in 37.5% ethylene glycol mono-methyl ether and 62.5% toluene. The (^{35}S)-methionine counts in each slice were determined using a Beckman LS-7500 liquid scintillation counter and the counts per minute in each gel slice were then plotted against gel slice numbers. The total number of counts under the peak representing TAT (53,000 daltons) were calculated.

Peptide Mapping by the Method of Cleveland

Phosphopeptides of TAT generated in vitro and in vivo were analyzed by the method of Cleveland et al. (1977). TAT was phosphorylated in vitro with PK-C as described. TAT phosphorylated in vivo for peptide analysis was generated with some modifications. Cells were grown to confluency in 100mm plastic plates and TAT synthesis was induced either by dexamethasone alone or by a combination of dexamethasone and 8-bromo-cyclic AMP. The reason that this procedure was used was that TAT concentrations in uninduced cells or cells induced with 8-bromo-cyclic AMP alone are too low to obtain sufficient

(^{32}P) incorporation to be useful. In addition, these cells were labeled with 10 mCi of (^{32}P i) instead of 2 mCi. All other conditions of labeling, incubation and harvesting were done as described above.

Immunoprecipitation of phospho-TAT was carried out as described above, however, preparation of the samples for electrophoresis involved some modifications. The pellet was dissolved in 160 μl of 10% SDS at 40°C for 2 hours and 40 μl of 5x stop was added before samples were boiled. Electrophoresis of 50 μl aliquots of each sample was carried out with 8.5% polyacrylamide slab gels as described above. The gels were stained for 20 minutes and destained for 45 minutes in the solutions described previously. The Coomassie-blue stained band representing both TAT and the heavy chain of IgG (these two do not separate on SDS gels since their molecular weights are close) were then excised from wet gels and equilibrated with Cleveland gel buffer (0.125M Tris, pH 6.8, 0.1% SDS, 1 mM EDTA) for 30 minutes.

The re-equilibrated gel slices were then placed into wells of a second polyacrylamide gel modified for peptide analysis. The composition of the running gel was 15% acrylamide and 0.396% bis-acrylamide and contained 0.5% linear polyacrylamide (Aldrich) in addition to the other components just described. The purpose of linear polyacrylamide is to prevent gels from cracking while drying. The composition of stacking gels was as described previously. Dimensions of these gels were also different—running gels were 12 to 14 cm long, stacking gels were 5 to 7 cm long with 2 cm deep wells and the gels were 2 mm thick. The purpose of the long stacking gel was to allow proteolysis to occur during stacking. The 2 mm thickness afforded easy loading of samples into the wells.

After TAT-containing gel slices were loaded into the wells of the second gel, 10 μ l of Cleveland gel buffer containing 20% glycerol was added to each well. Then 10 μ l of various concentrations of protease in Cleveland gel buffer containing 10% glycerol was added to each well. The proteases used were α -chymotrypsin (Sigma C-7762), papain (Sigma P-4762) and subtilisin (Sigma P-5380). All three proteases are capable of digesting protein during SDS-PAGE (Cleveland et al., 1977).

Electrophoresis was carried out using the running buffer solution described. In order for proteolysis to occur, the gels were run at 80 volts until the Coomassie-blue stain reached the junction of the running and stacking gel. The power was then shut off for 30 minutes so that proteolytic digestion could continue further. Electrophoresis was then continued at 180-200 volts until the Coomassie-blue dye front reached the bottom of the running gel. The running gel was then dried under heat and vacuum for 6 hours and autoradiographed as described above. No attempt was made to stain such gels.

Titration of Antibody to Carrier Tyrosine Aminotransferase

Specific antibody to tyrosine aminotransferase was prepared in rabbits using homogeneous TAT as prepared by rocket immuno-electrophoresis (Roper and Wicks, 1978) and was available in the laboratory. A crude preparation of carrier TAT was prepared up to the step of DEAE cellulose chromatography as described in the previous chapter. The enzyme was concentrated to 50,000 to 90,000 units per ml using a 3ml DEAE column eluted with buffer containing 0.3M potassium chloride. This concentrated material was dialysed against buffer containing 25mM potassium phosphate, pH 7.4, 25mM potassium chloride, 1.0mM EDTA, 0.2mM pyridoxal 5' phosphate, 5.0mM α -ketoglutarate and 10mM

β -mercaptoethanol and stored at -20°C in 300ml aliquots. Carrier TAT had a specific activity of 2000-4000 units per mg.

In order to standardize a particular preparation of carrier TAT with anti-TAT antiserum, an immunotitration was carried out. The enzyme was first diluted to 25,000 units per ml with phosphate-buffered saline, pH 7.4, and microfuge tubes (1.5ml) were set up in quadruplicate for six different amounts of TAT activity: 250, 500, 750, 1000, 1250 and 1500 units each. To two of each of these six samples was added 40 μl of antibody diluted with phosphate-buffered saline to give an antibody activity of 7500 units per ml. (A unit of antibody activity is that amount required to precipitate 1 unit of TAT activity.) The remaining samples were controls and contained 40 μl of phosphate-buffered saline instead of antibody. The final reaction volume of all samples was 100 μl . Each of the samples was incubated for 30 minutes at 37°C and 75 minutes at 4°C , then diluted to 1 ml by addition of phosphate-buffered saline and centrifuged in a Brinkman microfuge for 10 minutes. The supernatant was then assayed for TAT activity and this was expressed as total units in the 100 μl incubation mixture. A graph correlating TAT activity added to each sample and that remaining in the supernatant after incubation with and without antibody is shown in figure 3.2. The difference in TAT activity between the two lines is the amount of anti-tyrosine aminotransferase activity present in 40 μl of the diluted antibody.

Specificity of the Antibody

It is crucially important that the antibody used in these experiments exhibit high specificity for TAT. An experiment using two properties of this enzyme was designed to show that the antiserum used in this work is indeed

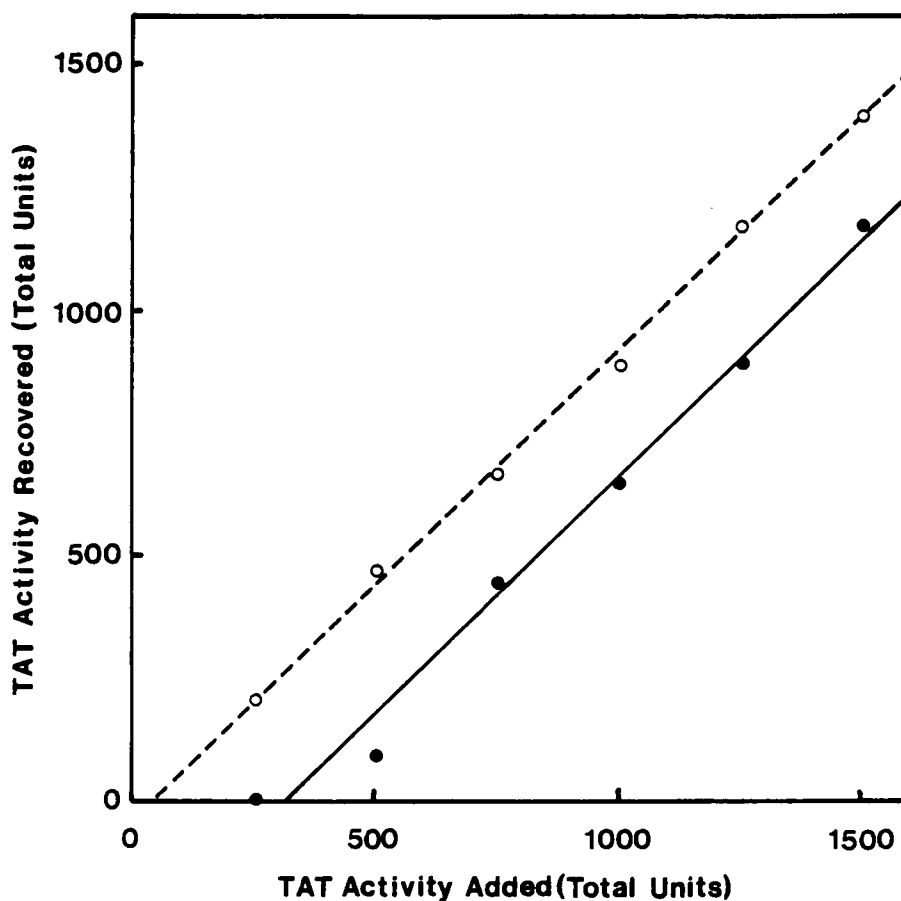


Figure 3.2. Titration of antibody to carrier TAT. The experiment was conducted as described in the text. 40 μ l of the 10x diluted antibody represented 260 units of TAT antibody activity. (●) Samples containing antibody and (o) samples without antibody.

highly specific. This experiment showed that the antibody reacted with a protein in a cell lysate that had a subunit molecular weight of 53,000 and that it was also inducible by dexamethasone. These are two known properties of TAT (Hargrove et al., 1980a; Wicks, 1974).

Figure 3.3 shows both a Coomassie blue stained slab gel and the resulting autoradiogram of (^{35}S)-methionine labeled TAT that was immunoprecipitated from dexamethasone-induced cells as described above. The gel was exposed to the X-ray film for 12 days with an enhancing screen at -70°C . The duration of this exposure was necessary in order to visualize all (^{35}S)-methionine labeled proteins in the gel, thus insuring that any minor proteins would be seen. The autoradiogram in figure 3.3 shows that most of the radioactivity is in a protein with a subunit molecular weight of 53,000 as calculated by its migration with protein standards. Very few other labeled proteins are apparent in the autoradiogram. This autoradiogram indicates the high degree of specificity of the antibody for TAT.

Figure 3.4 shows that this antibody also reacted specifically with a cellular protein that is inducible with dexamethasone and that the degree of increase in its synthesis is the same as the increase seen in TAT activity in this experiment. Thus, TAT activity in dexamethasone treated cells showed a 2.7 fold increase when compared to uninduced cells (64 units/mg lysate from dexamethasone treated cells versus 24 units/mg lysate from untreated cells). When a sample of each lysate was immunoprecipitated and analyzed by SDS-gel electrophoresis in tube gels, (figure 3.4) the vast majority of the radioactivity appeared in a protein with a subunit molecular weight of 53,000. The total counts per minute of (^{35}S)-methionine in gel slices 17-28 in the dexamethasone induced cells is 2.8 fold higher than those in the uninduced

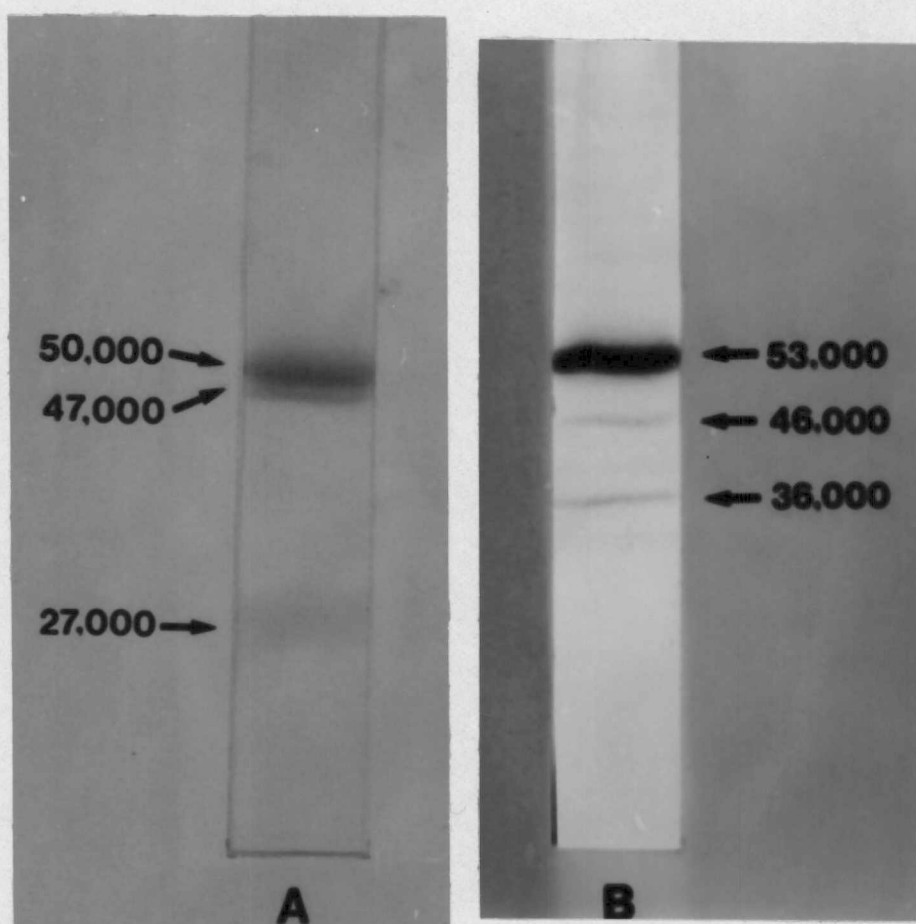


Figure 3.3. Coomassie blue-stained SDS-polyacrylamide gel (8.5%) (A) and autoradiogram (B) of (^{35}S)-methionine labeled TAT immunoprecipitated from dexamethasone induced H-35 cells. Protein bands representing the light and heavy chains of IgG (27,000 and 50,000) and carrier TAT (47,000) are the only proteins apparent in this gel. The autoradiogram, which was purposely overexposed as explained in the text, shows that the major amount of radioactivity is located in a protein with a molecular weight of 53,000. The lower molecular weight proteins which are apparent in the autoradiogram could be proteolytic fragments of TAT.

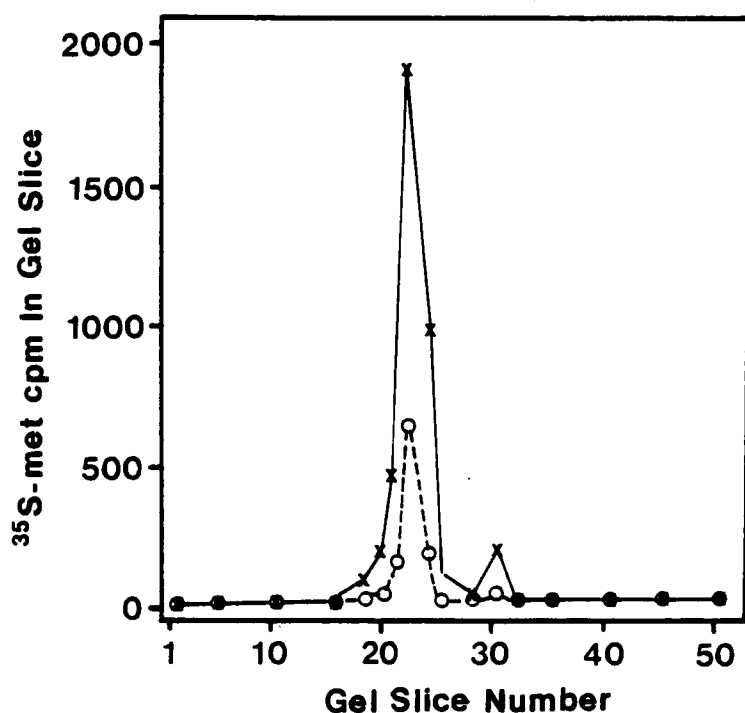


Figure 3.4. TAT antibody reacts specifically with a cellular protein inducible by dexamethasone and which has a molecular weight of 53,000. The experiment was performed as explained in the text. Dexamethasone treated cells (x) showed a 2.7 fold increase in TAT activity and a 2.8 fold increase in (³⁵S)-methionine counts immunoprecipitated at 53,000 daltons by anti-TAT antibody when compared to uninduced cells (o).

cells (3974 cpm versus 1382 cpm). The close correspondence in changes in catalytic activity and rate of synthesis argue strongly against the presence of a contaminating protein with a subunit molecular weight of 53,000 daltons. The data from this experiment indicate that the antibody preparation used in this work has a high degree of specificity for a protein from cell lysates with two well known properties of TAT.

CHAPTER IV

RESULTS

Evidence for In Vitro Phosphorylation of TAT by PK-C

Figure 4.1 is an autoradiogram of in vitro phosphorylation reactions designed to show that PK-C phosphorylates one major protein in the TAT preparation that is immunoprecipitated by anti-TAT antibody. The PK-C preparation, which in this instance was approximately 70% pure, contains a number of phosphorylated substrates including itself (lane 1); however only trace amounts of phosphoprotein are non-specifically trapped after immunoprecipitation with anti-TAT antibody (lane 2). The TAT preparation, which was approximately 50% pure, does not possess any detectable kinase activity (lanes 3 and 4). When TAT and PK-C are incubated together, only one additional protein with a molecular weight of 53,000 exhibits substantial phosphorylation (lane 5) and this protein is precipitated by anti-TAT antibody (lane 6).

Control of Factors Influencing Stoichiometry Determinations

It is important that the in vitro phosphorylation of TAT by PK-C be completely stopped before immunoprecipitation. If the reaction is not completely stopped, crude carrier TAT added during immunoprecipitation is phosphorylated and gives false phosphate incorporation determinations.

Addition of 50 mM EDTA has been found to effectively stop the phosphorylation reaction (figure 4.2). Phosphorylation of all substrates including TAT by PK-C is abolished in the presence of EDTA (lane 1 and 2), in contrast to the situation when EDTA is not present (lanes 3 and 4). Lane 4, in which TAT is immunoprecipitated from a phosphorylation reaction to which EDTA

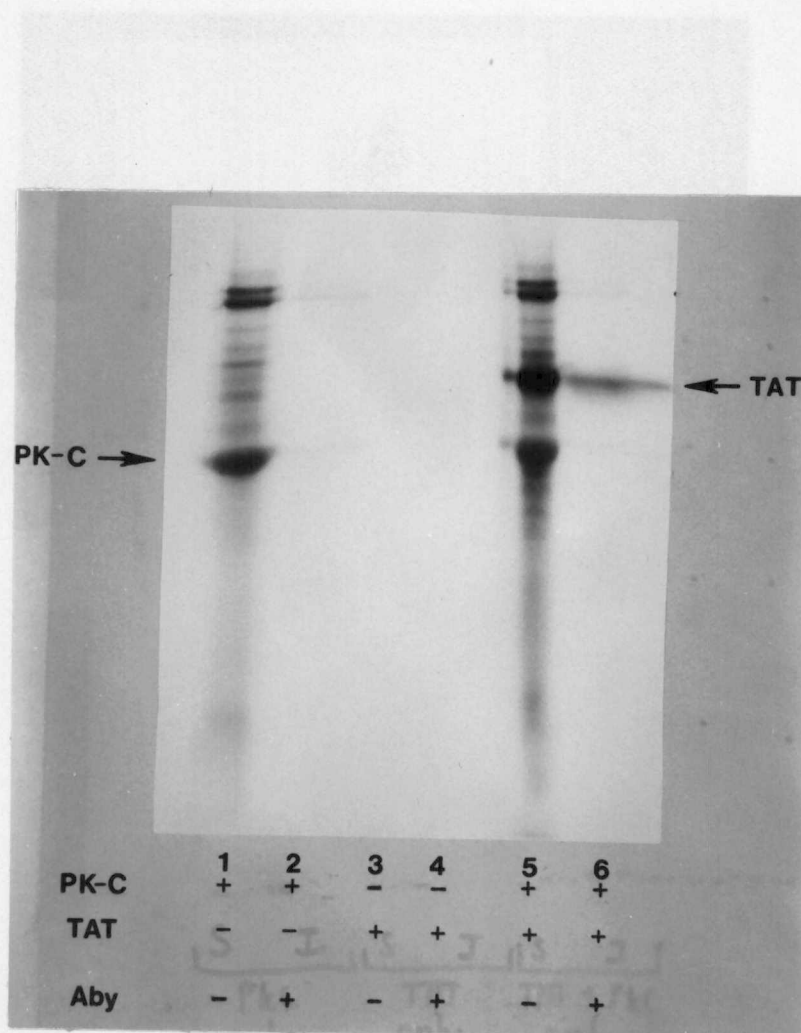


Figure 4.1 Autoradiogram of phosphorylation reactions. Phosphorylation reactions were carried out with PK-C alone (lanes 1 and 2), TAT alone (lanes 3 and 4) and both PK-C and TAT (lanes 5 and 6). In each case, 144 pmoles of PK-C (6.7 units/mg; 70% pure) and 5.5 pmoles of TAT dimer (161,000 units/mg; 50% pure) were used. Each of the reactions was analyzed by SDS-PAGE with and without immunoprecipitation with specific antibody (Aby).

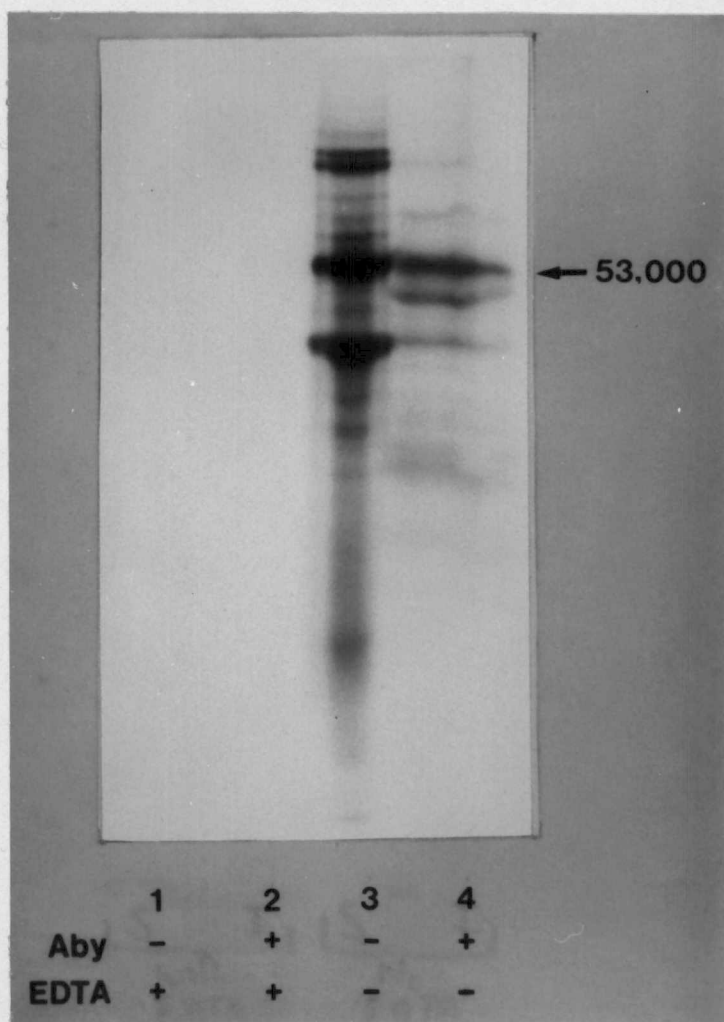


Figure 4.2. Autoradiogram showing that EDTA effectively halts the phosphorylation of TAT by PK-C. Lanes 1 and 2 show a phosphorylation reaction carried out in the presence of 50 mM EDTA. Lanes 3 and 4 show the same reaction without EDTA. 11 pmoles of TAT dimer (161,000 units/mg; 50% pure) was phosphorylated with 144 pmoles PK-C (6.7 units/mg; 70% pure) with 200 μ M γ -(^{32} P)-ATP for 30 minutes. Each of the reactions was analyzed using SDS-PAGE with and without immunoprecipitation by specific antibody (Aby).

was never added, shows that several phosphoproteins with molecular weights below that of TAT are present. They were not seen when EDTA was added to the incubation mixture before the antibody and carrier TAT were added (figure 4.1, lane 6). These phosphoproteins are probably proteolytic fragments of TAT contained in the crude carrier preparation. These fragments are expected to be present since carrier TAT preparations are not subjected to CM-Sephadex C-50 chromatography.

Phosphate incorporation into TAT could be significantly decreased if the anti-TAT antibody or the crude carrier TAT preparation contained phosphatase activity. Sodium fluoride and sodium pyrophosphate are known to inhibit activity of phosphatases and are in standard use for this purpose (Aswad and Greengard, 1981). Table 4.1 shows that addition of 10 mM sodium fluoride or 2 mM sodium pyrophosphate to the antibody or carrier TAT did not significantly change the amount of phosphate incorporated into TAT by PK-C. It was concluded that neither the antibody nor the crude carrier preparation contained phosphatase activity which could be inhibited by standard methods.

Phosphatase activity may also be found in cell lysates. It is possible that upon lysis of cells, phosphatases are released which immediately dephosphorylate cellular proteins. Table 4.2 shows that when 10 mM sodium fluoride or 2 mM sodium pyrophosphate are included in the lysis buffer, there is no change in the amount of phosphate incorporated into TAT isolated from cells.

Table 4.1

Evidence of Absence of Inhibitable Phosphatase Activity in Anti-TAT Antiserum
and Crude Carrier TAT Used for Immunoprecipitation

Sample	cpm/53,000 gel band*
Phosphate buffered saline	1500 $\pm$ 300
10 mM NaF	1600 $\pm$ 300
2 mM Na pyrophosphate	1700 $\pm$ 400

*Data are average of three determinations $\pm$ standard deviation.

Table 4.2
Evidence of Absence of Inhibitable Phosphatase
Activity in Cell Lysates.

Sample	cpm/total TAT activity*		
Phosphate buffered saline	23	$\pm$	2
10 mM NaF	23	$\pm$	5
2 mM Na pyrophosphate	27	$\pm$	4

*Data are average of three determinations $\pm$ standard deviation.

Optimization of Reaction Conditions

Having confirmed Stellwagen's report that TAT can be phosphorylated in vitro by PK-C, an attempt was made to optimize the reaction conditions. Stellwagen and Kohli (1981) did not report any effort to modify the conditions for in vitro phosphorylation of TAT and they obtained only modest incorporation of (^{32}P) (0.2 moles of phosphate per mole of TAT). Figure 4.3 illustrates changes in the degree of TAT phosphorylation as a function of ATP concentration during a 30 minute reaction at 30°C. The total radioactivity added in each sample was kept constant. Phosphate incorporation was maximum at 200-400 μM ATP; therefore 200 μM ATP was chosen as the final ATP concentration for all in vitro phosphorylation reactions.

Figure 4.4 shows that phosphorylation of TAT by PK-C was essentially complete by 30-60 minutes when a ratio of PK-C to TAT dimer of about 7:1 was used (see below). About 0.7 moles of phosphate was incorporated into each mole of TAT subunit when the reaction began to level off which is substantially higher than the 0.2 moles obtained by Stellwagen and Kohli (1981).

Figure 4.5 shows that maximum phosphate incorporation into TAT occurred at a PK-C to TAT ratio of 7:1 during a 30 minute reaction. Although this ratio seems high, the ratio of PK-C to TAT in rat liver has been calculated to be 10:1 (Boney et al., 1983). Therefore, this in vitro ratio appears to be within the physiological range and was used to obtain maximum phosphorylation of TAT. Since a maximum of 0.7 moles of phosphate was incorporated into each TAT subunit in 30 to 60 minutes at 30°C with this mole ratio, 30 to 60 minute reaction times are used throughout this work.

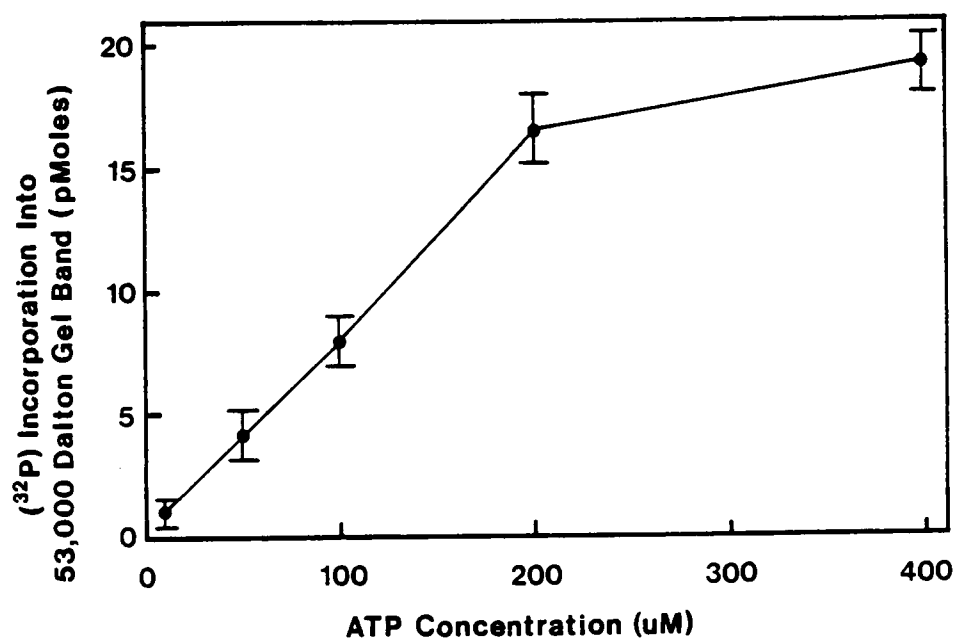


Figure 4.3 Effect of ATP concentration on phosphorylation of TAT by PK-C. 263 pmoles PK-C (4.2 units/mg; 50% pure) were used to phosphorylate 18 pmoles TAT dimer (159,000 units/mg; 30% pure). The specific activity of the γ -(³²P)-ATP was 1000 cpm/pmole in each case. Phosphate incorporation was determined by immunoprecipitation and SDS-PAGE. Results are the average of two experiments.

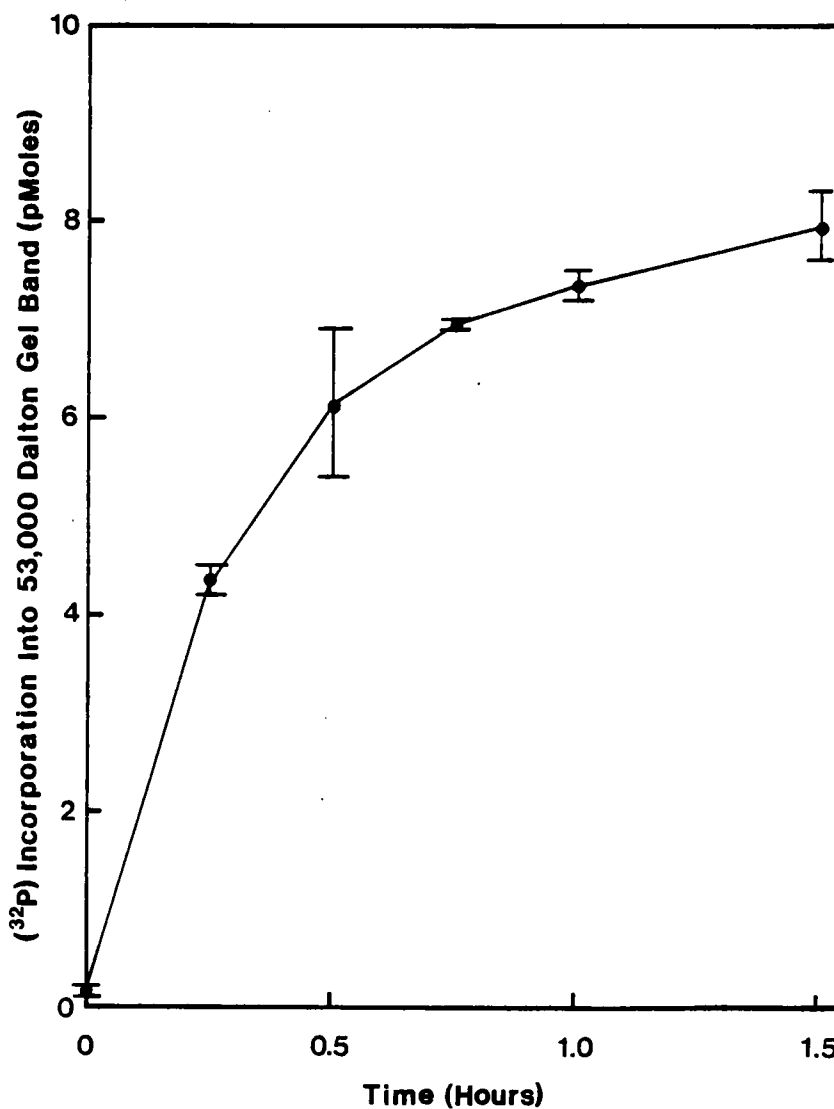


Figure 4.4. Kinetics of phosphorylation of TAT by PK-C. 36 pmoles PK-C (6.7 units/mg; 70% pure) were used to phosphorylate 5.5 p moles of TAT dimer (161,000 units/mg; 50% pure). Reactions were carried out for the indicated time periods at 30°C. The γ -(^{32}P)-ATP concentration was 200 μM in each case. Phosphate incorporation was determined by immunoprecipitation and SDS-PAGE. Results are the average of two experiments.

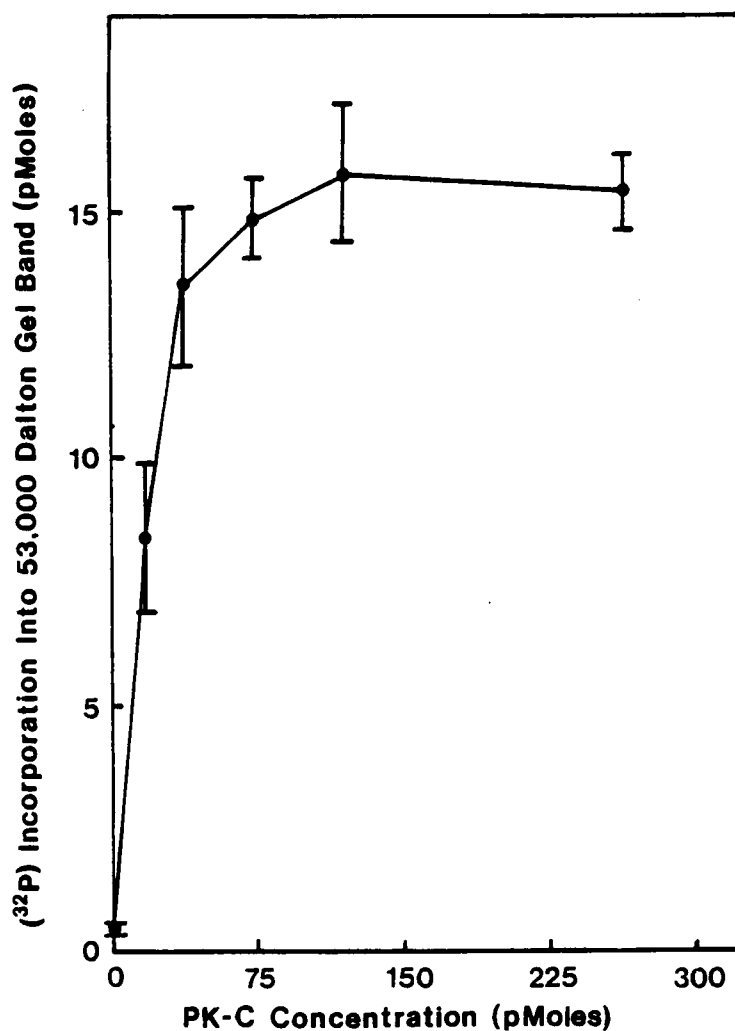


Figure 4.5. Effect of PK-C concentration on TAT phosphorylation. Various PK-C concentrations (6.7 units/mg; 70% pure) were used to phosphorylate 11 pmoles of TAT dimer (161,000 units/mg; 50% pure). Reactions were carried out for 30 minutes at 30°C with 200 μ M γ -(³²P)-ATP. Phosphate incorporation was determined by immunoprecipitation and SDS-PAGE. Results are the average and standard deviation of three experiments.

Comparison of Sites of Phosphorylation In Vivo and In Vitro

Different concentrations of three proteases, α -chymotrypsin, papain and subtilisin, gave various phospho-peptide patterns as shown by autoradiography of Cleveland gels (Figure 4.6). As expected, increasing protease concentrations produced lower molecular weight phosphopeptides. ATP does not remain on these gels during electrophoresis as shown when 40,000 cpm of γ -(^{32}P)-ATP were loaded (data not shown). In addition, none of the samples showed degradation when protease was not added to the gel (data not shown).

Artifacts are apparent in these autoradiograms. These artifacts involve cracks in the dried gel. It is important that these are properly noted so that a single peptide spot in an autoradiogram is not accidentally interpreted as representing two peptides.

Figures 4.7 and 4.8 show autoradiograms in which the peptide maps of phospho-TAT prepared in vitro and in vivo were compared. Both autoradiograms were prepared under similar conditions, except that protease concentrations used in figure 4.8 were higher than those used in figure 4.7. Peptide maps of TAT isolated from H-35 cells induced with dexamethasone (A) or both dexamethasone and 8-bromo-cyclic AMP (C) were similar; however TAT phosphorylated in vitro by PK-C had, in some cases, a very different peptide map. The difference between in vivo and in vitro phospho-TAT peptides is most striking with α -chymotrypsin. The map produced with papain showed less striking differences and the subtilisin maps were rather similar.

The in vivo α -chymotrypsin maps (lanes A and C) in figure 4.7 show phospho-peptide patterns which seem to be identical, indicating that 8-bromo-cyclic AMP does not alter this pattern. Both lanes A and C contain phosphopeptides with high molecular weights (R_f 's of 0.1-0.2) and they also contain

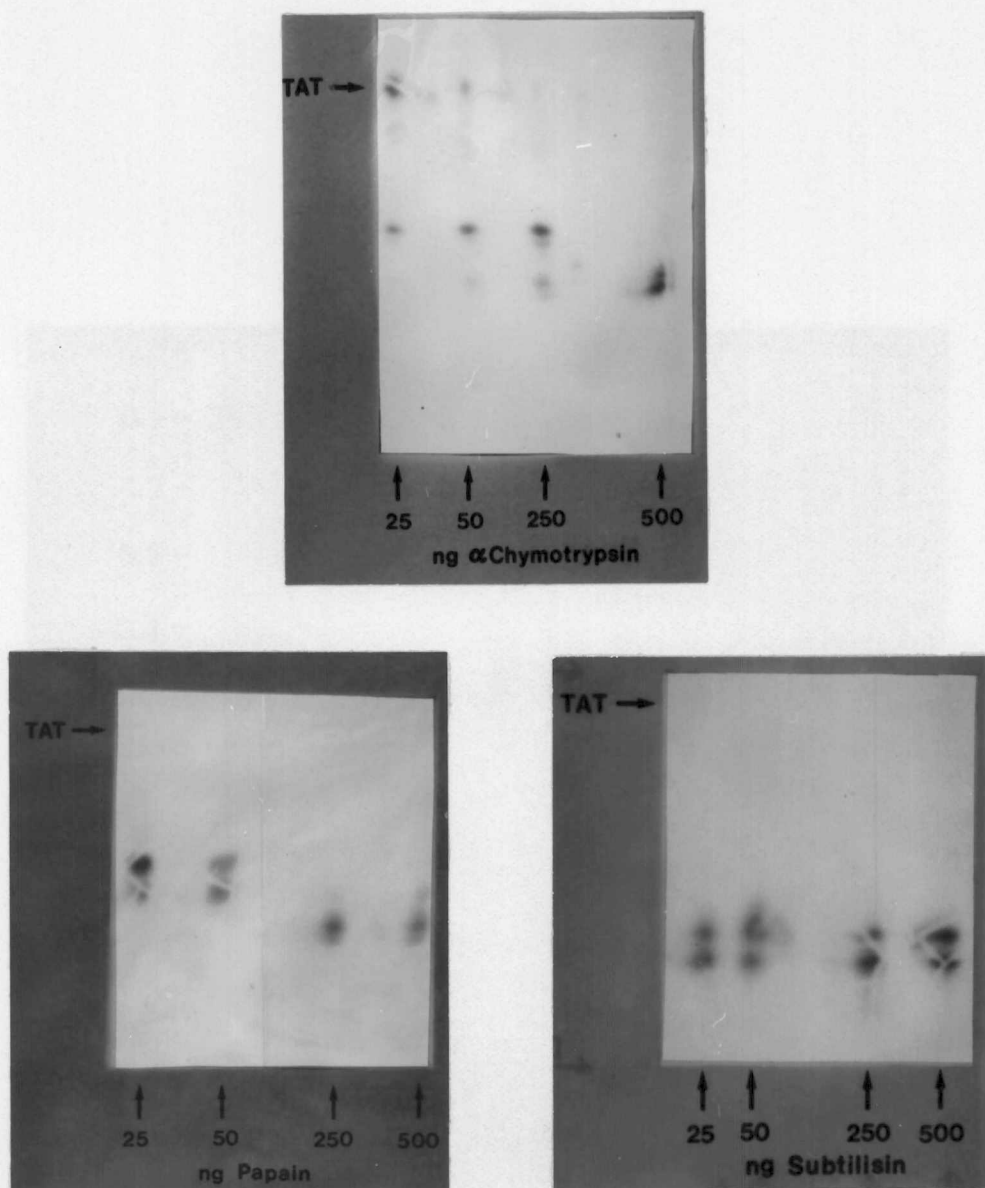


Figure 4.6. Autoradiograms of phospho-peptide maps of TAT phosphorylated in vitro by PK-C. The indicated concentrations of α -chymotrypsin, papain and subtilisin produced various phospho-peptide maps by the method of Cleveland et al. (1977) as described in Materials and Methods.

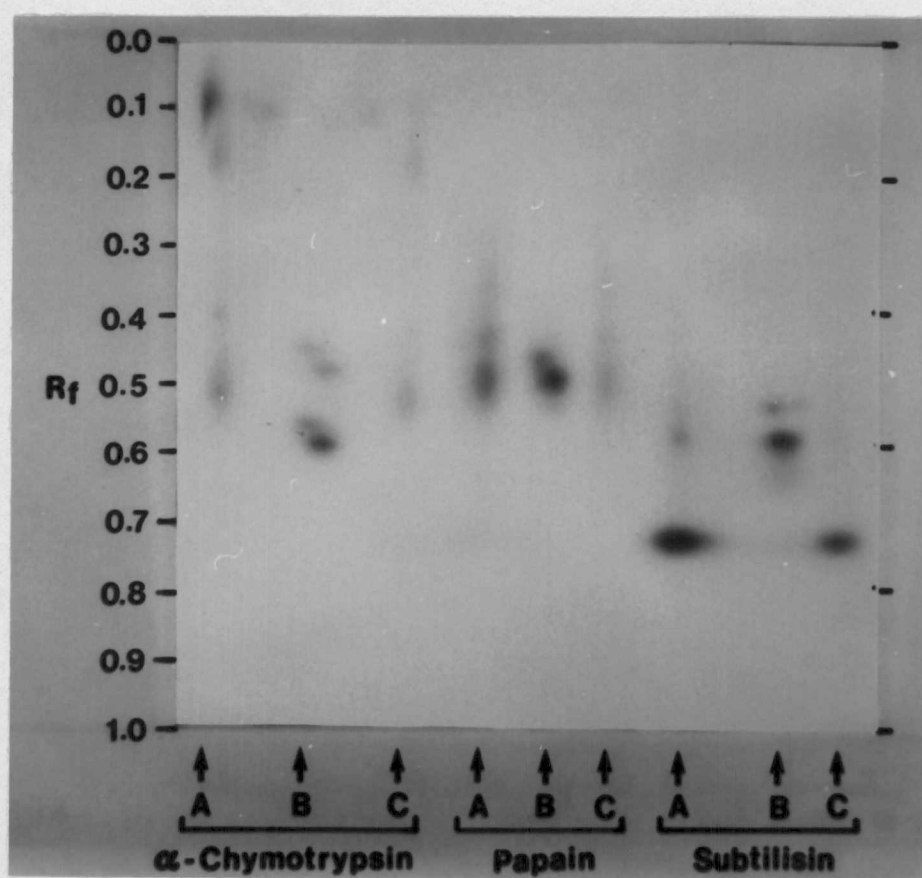


Figure 4.7. Autoradiogram of phospho-peptide maps of TAT phosphorylated *in vitro* by PK-C (B) and phospho-TAT immunoprecipitated from H-35 cells induced with dexamethasone alone (A) or dexamethasone with 8-bromo-cyclic AMP (C). Digests were done by the method of Cleveland *et al.* (1977) with 0.750 μg α -chymotrypsin, 0.500 μg papain or 0.500 μg subtilisin.

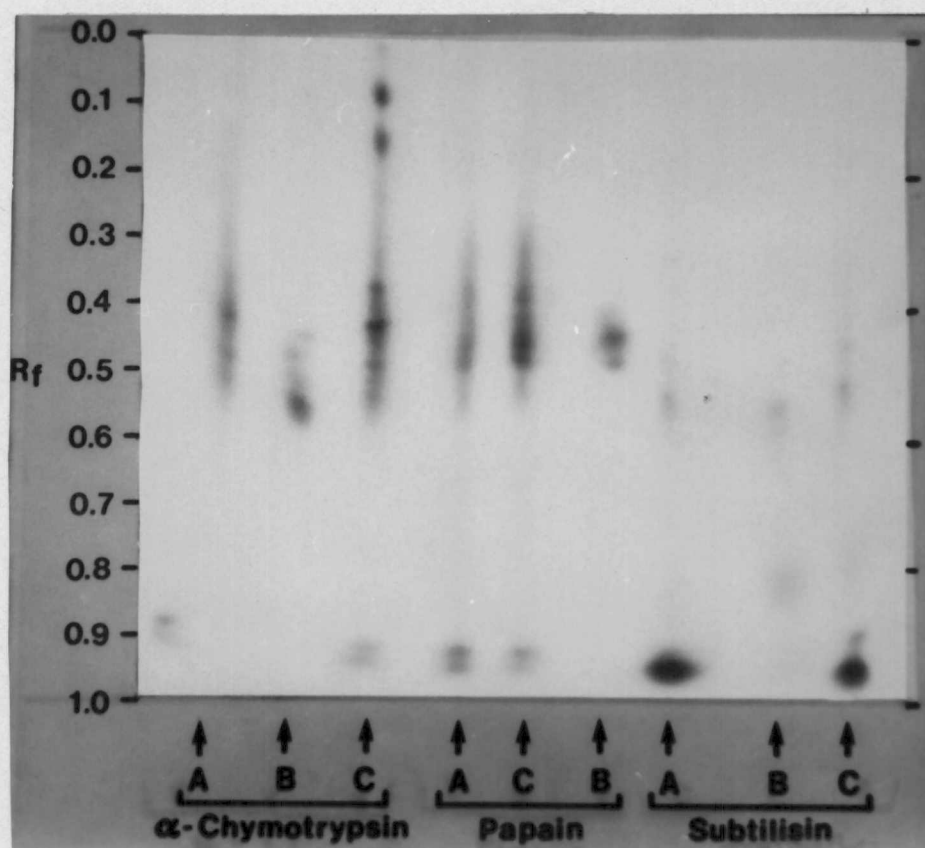


Figure 4.8. Autoradiogram as in figure 4.7 using higher protease concentrations. In this experiment, all protease concentrations were 1.0 μ g. This autoradiogram was exposed without enhancing screens.

two phospho-peptides with R_f 's of approximately 0.4 and 0.5. The in vitro map produced by α -chymotrypsin (lane B) does not contain any high molecular weight phospho-peptides, but does contain four phospho-peptides with R_f 's in the range of 0.5 and 0.6. These in vitro phospho-peptides seem to have a different mobility than those derived from in vivo sources.

In order to confirm this result, the experiment was repeated using higher α -chymotrypsin concentrations (figure 4.8). The in vivo lanes (A and C) still showed high molecular weight material (R_f 's 0.1-0.2) and the phospho-peptides in the 0.4-0.5 R_f range. The in vitro map (lane B) still had approximately four phospho-peptides whose R_f 's were similar to those found in figure 4.7. This indicated that a higher α -chymotrypsin concentrations did not change the in vitro pattern significantly. The in vivo α -chymotrypsin maps (lanes A and C) of figure 4.8 have some phospho-peptides with R_f 's of 0.4-0.5, two of which may have the same mobility as α -chymotrypsin phospho-peptides derived from in vitro sources. However, the in vivo maps also contained a pair of phospho-peptides of low molecular weight which were not seen with the in vitro material (R_f 's of about 0.9).

Both in vivo papain maps (figure 4.7, lanes A and C) have identical phospho-peptide patterns, again indicating that 8-bromo-cyclic AMP had no effect on which peptides contained phosphate. However, differences from the in vitro map are not as striking with papain as they were with α -chymotrypsin. Both in vivo and in vitro papain maps contain phospho-peptides with R_f 's of 0.4-0.5. The major difference between the in vivo and in vitro papain maps in figure 4.7 is that the in vivo maps contain phospho-peptides in the 0.3 and 0.4 R_f range while the in vitro map does not.

In order to see if the differences noted in the papain maps in figure 4.7 were reproducible, the experiment was repeated using a two-fold higher papain concentration (figure 4.8). In this experiment, the in vivo papain maps (lanes A and C) still showed material with R_f 's of 0.2-0.4, which were not in the in vitro papain map (lane B). All three maps still contained material with R_f 's of 0.4-0.5, however, the in vivo maps also contained a pair of phospho-peptides with R_f 's greater than 0.9 which do not appear in the in vitro map. Therefore, although there are some papain phospho-peptides whose mobilities may be the same both in vivo and in vitro, the overall pattern and the mobilities of many individual papain phospho-peptides are different from the two sources.

The use of subtilisin produced in vivo and in vitro phospho-peptide maps which were quite similar. In figure 4.7, subtilisin phosphopeptides derived from cells not treated with 8-bromo-cyclic AMP (lane A) contained phospho-peptides with R_f 's of about 0.5. These phospho-peptides were apparent in the in vitro subtilisin map (lane B), but were not apparent in the map from cells treated with 8-bromo-cyclic AMP (lane C). However, since these radioactive spots in lane A of the subtilisin map were only barely visible, one can not be sure if these spots did not show up in lane C because the amount of radioactivity was too low.

The in vivo and in vitro maps produced by subtilisin in figure 4.7 were different with respect to low molecular weight phospho-peptides found in vivo with R_f 's of 0.7 that were not apparent in vitro. To be sure that this difference was not due to incomplete digestion of the in vitro sample, the experiment was repeated using double the subtilisin concentration (figure 4.8). In this experiment, the in vivo subtilisin maps (lanes A and C) showed phospho-peptides with R_f 's of almost 1.0. These phospho-peptides were not apparent in the in

vitro lane until the Cleveland gel was exposed to X-ray film with an enhancing screen (figure 4.9). Enhancing also showed that there were some subtilisin phospho-peptides with R_f 's of about 0.3-0.4 from both in vivo and in vitro sources. (Enhancing of the Cleveland gel in figure 4.9 did not cause visualization of any additional phospho-peptides in the α -chymotrypsin or papain maps that were not already seen in figure 4.8). Therefore subtilisin produces similar phospho-peptide maps irrespective of the source of TAT used in this work.

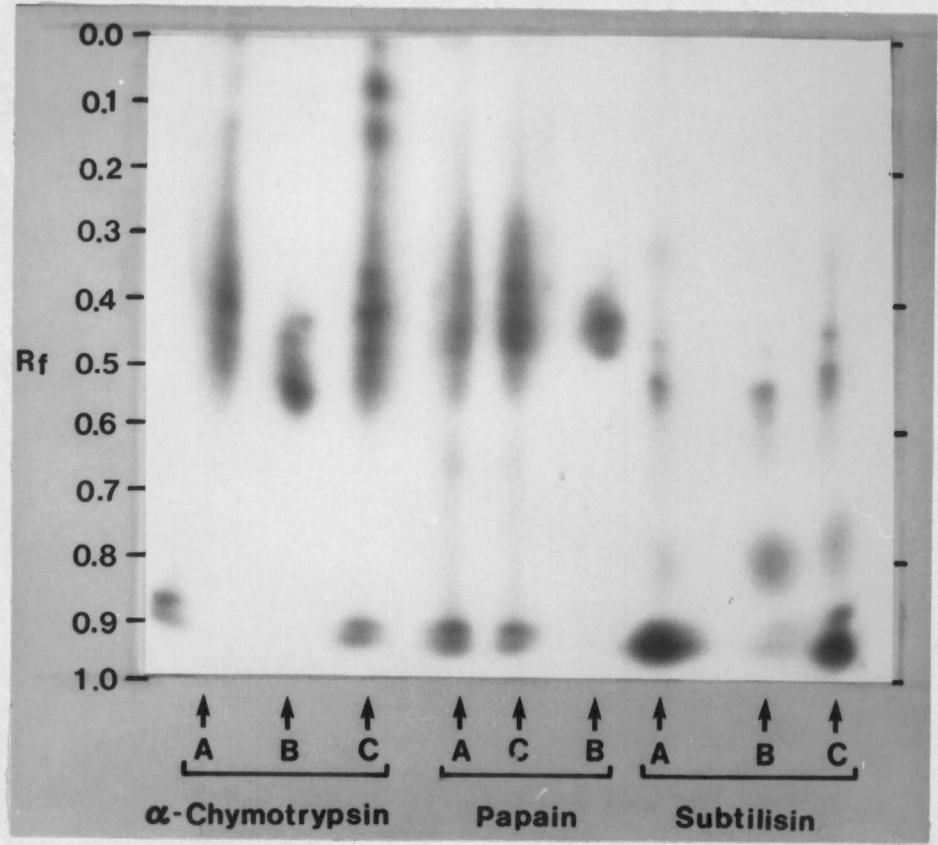


Figure 4.9. Autoradiogram from the same Cleveland gel in figure 4.8 exposed with an enhancing screen.

CHAPTER V

DISCUSSION

Optimization of the reaction conditions allowed for a substantial increase in the degree of phosphorylation of TAT by PK-C in vitro over that reported by Stellwagen and Kohli (1981). Rough estimates show that up to 0.7 moles of phosphate was incorporated per mole of TAT subunit. Incorporation of 1 mole of phosphate per mole of subunit has not been observed in any experiment to date, however the extent of endogenous phosphate already in the TAT used was not known and experiments with phosphatase treated enzyme have not yet been tried. The presence of some unlabeled phosphate in the purified enzyme would not be unreasonable (Krebs and Beavo, 1979; Roach, 1981) and would suggest that more phosphate could be incorporated into the enzyme.

The reaction conditions used in this work to phosphorylate TAT in vitro were different from those used previously by Stellwagen and Kohli (1981). The most important change was that the final concentration of ATP used in the current work was 200 μM instead of the 10 μM concentration used by Stellwagen. This change significantly increased the amount of phosphate that could be incorporated into TAT. The use of higher ATP concentrations may explain why Stellwagen observed incorporation of only 0.2 moles phosphate per mole of TAT subunit and also why they were never able to achieve completion of phosphate incorporation during time course studies. The use of this ATP concentration is well within the range of ATP concentrations used by other investigators for in vitro phosphorylation reactions: 7 μM and 50 μM (Huttner et al., 1981); 400 μM (McPherson et al., 1979); 500 μM (Pierce et al., 1981); 100 μM (Ramakrishna and Benjamin, 1981); 140 μM (Van den Berg et al., 1980).

Since higher ATP concentrations caused more phosphate to be incorporated into TAT, other reaction conditions were adjusted to reflect this improved incorporation. The reaction time was decreased from 1.5 hours to 0.5 hours, where essentially maximal phosphorylation occurred with 200 μ M ATP. Additionally, PK-C was added only at the beginning of the reaction instead of three times during the course of the reaction as in Stellwagen's experiments.

The in vitro phosphorylation reaction conditions used in these experiments were not typical of an enzyme-catalyzed reaction. Peak incorporations of phosphate into TAT was reached when PK-C concentrations were 7 fold higher than TAT concentrations. Usually, it is expected that the opposite situation would occur, where the catalyst, PK-C, is used in very small concentrations compared to substrate. Although the levels of PK-C were high, this was rationalized by the levels of PK-C and TAT found in rat liver where PK-C concentrations are calculated to be 10 fold higher than TAT concentrations (Boney et al., 1983). So, even though the in vitro conditions indicate that the phosphorylation reaction may be occurring under forced conditions, it did occur within calculated physiological ratios.

In all phosphorylation reactions described in this work, the undegraded form of TAT (type I) as described by Hargrove et al. (1980a) was used exclusively. Stellwagen used TAT preparations that consisted of only 20% undegraded subunit, which means that most of his preparation consisted of type II and type III TAT. Actually, it is possible that the preparations employed by Stellwagen and Kohli (1981) contained no or very little type I TAT since all 20% of the undegraded subunit could have conceivably come from type II TAT dimer. (Stellwagen used the method of Valeriote et al., 1969 to purify TAT. This method does not include precautions which prevent proteolysis of

TAT by cathepsin T during homogenation, nor does it employ a method to separate the undegraded form from other forms of the enzyme.) Therefore, it is felt that the in vitro phosphorylation experiments described in the present work more accurately simulate the potential phosphorylation conditions in vivo (i.e., where the only form of TAT is type I, the ATP concentration is in the millimolar range and where the concentration of PK-C is higher than TAT).

A major problem in working with TAT in these experiments was that the enzyme was not stable. This not only meant that experiments had to be done soon after TAT was prepared if catalytically active enzyme was to be used, but also that the actual ratio of catalytically active to inactive TAT was not known. Specific activities of preparations did not provide insight into this problem since a purified preparation of the enzyme exhibits continually decreasing specific activities with time and since different preparations of enzyme with vastly different degrees of purity exhibited similar specific activities (see figures 2.5 and 2.6). Since the mechanism of activity loss is not understood, no rigorous assessment of how this inactivation influenced the phosphorylatability of TAT by PK-C could be made.

It is very possible that catalytically active TAT only represents a small percentage of the total TAT present in any given preparation and that this active TAT was never phosphorylated. Knowing whether or not the inactivation of TAT makes it more susceptible to phosphorylation is a very important question since many enzymes are more susceptible to phosphorylation when they are denatured (Bylund and Krebs, 1975). Preliminary work on this problem has indicated that fresh, active preparations of TAT which are inactivated by freezing and thawing are poor substrates for PK-C. (80 % less phosphate was incorporated into TAT that was inactivated more than 99% by freezing and

thawing as compared to TAT which was kept at 4°C and had no loss in activity). However, TAT preparations which are stored over a long time period and have lost catalytic activity are still good substrates for PK-C. The final answer to this apparent dilemma can not be properly explored until a method is found that will separate catalytically active TAT from inactive forms of the enzyme.

Peptide mapping results strongly suggest that PK-C is not responsible for phosphorylation of TAT in vivo. This is because phosphorylation of a protein by PK-C in vivo should occur in intact cells in response to an appropriate stimuli at the same sites phosphorylated by PK-C in vitro (Cohen, 1980). Peptide mapping results with α -chymotrypsin show that PK-C, in vitro, does not phosphorylate the same sites in TAT that are phosphorylated in cells treated with 8-bromo-cyclic AMP. 8-bromo-cyclic AMP is known to activate PK-C in the cell line used for this work (Wimalasena and Wicks, 1979). If PK-C could phosphorylate TAT in vivo, then one would expect some similarity between the sites phosphorylated in vitro by PK-C and those sites phosphorylated in intact cells treated with 8-bromo-cyclic AMP; this was not so.

There are, however, two phospho-peptides in the α -chymotrypsin map that may have had the same mobilities both in vivo and in vitro (figure 4.8). Assuming these peptides were the same peptides (and not different peptides of similar molecular weight) and were phosphorylated on the same sites, this might mean that PK-C is weakly phosphorylating a site in vivo. These peptides were seen in vivo regardless of whether or not 8-bromo-cyclic AMP was added to the cells. This may indicate that PK-C did not phosphorylate TAT in vivo since this site was phosphorylated in vivo without 8-bromo-cyclic AMP being added to the cells. (PK-C is not active in these cells when 8-bromo-cyclic

AMP is not present (Wimalasena and Wicks, 1979) and therefore substrates of PK-C in these cells would not be expected to be phosphorylated.) However, one can not rule out the possibility that PK-C and a second kinase both phosphorylate the same site within TAT. At least three such examples are known. Synapsin I and protein III are two neuronal phosphoproteins which are both phosphorylated on the same site by PK-C and calcium/calmodulin I protein kinase (Nestler and Greengard, 1983). Also, glycogen synthase from muscle is phosphorylated by three different kinases on one particular site (Cohen, 1982). Unfortunately, the complete nature of these minor sites in TAT can not be fully understood until a highly quantitative study of the stoichiometry and sequence of phosphorylation sites is done.

Although the peptide map produced by α -chymotrypsin indicated that PK-C was not responsible for the phosphorylation of TAT in vitro, the subtilisin map indicated that the phosphopeptides could be similar. This observation suggests that the PK-C phosphorylation site(s) in vitro is in the same subtilisin-sensitive region of TAT as the in vivo site(s). However, since the α -chymotrypsin map showed the sites were different, it is reasonable to believe that the phosphorylated site(s) within the subtilisin-sensitive peptides are different in vivo and in vitro.

The papain map is, in some respects, the most interesting. This is because it contained some phospho-peptides whose mobilities may be the same both in vivo and in vitro as well as containing phospho-peptides whose mobilities are different. These data might be suggestive of two sites of phosphorylation in vivo. One in vivo site may be on the same papain-sensitive peptide as an in vitro site (but on a different place within the peptide), while the other in vivo site is on a papain-sensitive site that is not represented in vitro. Although

this is highly speculative, the work done by Stellwagen and Kohli (1981) showed that there are two tryptic phosphopeptides from TAT phosphorylated in vivo which would also be consistent with two sites of phosphorylation.

With respect to the relationship between in vivo and in vitro sites of TAT phosphorylation, the results of this work contrast with those presented by Stellwagen and Kohli (1981). Their peptide mapping data indicated that the site of phosphorylation in vitro by PK-C is the same as one of the two proposed in vivo sites. The data presented in this work is not in agreement. One possible reason for the discrepancy is that Stellwagen and Kohli used trypsin while the definitive data in this work was obtained using α -chymotrypsin. Since the specificities of these two proteases are different, the mapping results may be different because the in vitro and in vivo phosphorylation sites may be on the same tryptic peptides, but on different chymotryptic peptides.

The discrepancy may also be due to phosphate incorporation into TAT by PK-C in vitro that was approximately 3.5 fold higher in this work than in Stellwagen and Kohli's. It is possible that Stellwagen did not phosphorylate all potential phosphorylation sites in vitro thereby missing potential in vivo and in vitro differences. This might also explain why the α -chymotrypsin map indicated that two minor phospho-peptides may have been common to both in vivo and in vitro phosphorylated TAT while the rest of the map was very different.

A final question arising from this work is why PK-C is able to phosphorylate TAT in vitro but apparently not significantly in vivo. One possibility is that TAT isolated for in vitro phosphorylations is not in the same conformation as it is found in vivo and this conformational difference is exposing sites in vitro that would not be exposed in vivo (Bylund and Krebs,

1978). Another possibility is that since this work was done with highly purified enzymes, TAT did not have to compete with other PK-C substrates in vitro, but does in vivo. A comparison of the K_m of PK-C for TAT and other PK-C substrates from liver would help answer this question.

The work presented in this thesis indicates that PK-C is probably not responsible for the majority of TAT phosphorylation in vivo. This conclusion agrees with earlier results of Wicks and Su (1978) which indicated that an increase in the amount of phosphate incorporated into TAT in vivo did not require activation of cAMP-PK. The next stage of this work should involve an attempt to isolate the actual kinase responsible for the phosphorylation of TAT.

LIST OF REFERENCES

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- Aswad, D.W. and Greengard, P. (1981) *J. Biol. Chem.* 256, 3494-3500.
- Aviram, M. and Hershko, A. (1977) *Biochim. Biophys. Acta* 498, 83-90.
- Barnett, C.A. and Wicks, W.D. (1971) *J. Biol. Chem.* 246, 7201-7206.
- Bechtel, P.H., Beavo, J.A. and Krebs, E.G. (1977) *J. Biol. Chem.* 256, 2691-2697.
- Boney, C., Fink, D., Schlichter, D., Carr, K. and Wicks, W.D. (1983) *J. Biol. Chem.* 258, 4911-4918.
- Buckley, W.T. and Milligan, L. (1978) *Biochem. J.* 176, 449-454.
- Bylund, D.B. and Krebs, E.G. (1978) *J. Biol. Chem.* 250, 6355-6361.
- Carr, R.K. (1984) Investigation of the in vitro Phosphorylation of Gamma Aminobutyrate Aminotransferase by cAMP Dependent Protein Kinase. Ph.D. dissertation. Univ. of Tenn., Knoxville.
- Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) *J. Biol. Chem.* 252, 1102-1106.
- Cohen, P. (1979) *Curr. Top. Cell. Regul.* 14, 117-196.
- Cohen, P. (1980) in *Recently Discovered Systems of Enzyme Regulation* (Cohen, P. Ed.) pp. 1-10.
- Cohen, P. (1981) *Adv. Cyclic Nucleotide Res.* 14, 345-359.
- Cohen, P. (1982) *Nature* 296, 613-620.
- Cohen, P., Foulkes, J.G., Goris, J., Hemmings, B.A., Ingebritsen, T.S., Stewart, A.A. and Strada, S.T. (1981) in *Metabolic Interconversions of Enzymes 1980* (Holzer, H. Ed.) pp. 28-43.
- Corbin, J.D., Sugden, P.H., West, L., Flockhart, C.A., Lincoln, T.M., and McCarthy, D. (1978) *J. Biol. Chem.* 253, 3997-4003.
- Diamondstone, T.I. (1966) *Anal. Biochem.* 16, 395-401.
- DiCola, D. and Federici, G. (1982) *Biochem. J.* 205, 265-269.
- Garibaldi, G. (1977) *Helv. Pediatr. Acta.* 32, 173-180.
- Gibco Catalogue: Tissue Culture Products (1982) Gibco Laboratories, Grand Island, N.Y. pp 150.

- Greengard, P. (1978) *Science* 199, 146-152.
- Gohda, E. and Pitot, H.C. (1980) *J. Biol. Chem.* 255, 7371-7379.
- Gohda, E. and Pitot, H.C. (1981) *J. Biol. Chem.* 256, 2567-2572.
- Hargrove, J.L., Diesterhaft, M., Noguchi, T. and Granner, D.K. (1980a) *J. Biol. Chem.* 255, 71-78.
- Hargrove, J.L. and Granner, D.K. (1980b) *Anal. Biochem.* 104, 231-235.
- Hargrove, J.L., Gohda, E., Pitot, H.C. and Granner, D.K. (1982) *Biochemistry* 21, 283-289.
- Hayashi, S., Granner, D.K. and Tomkins, G. (1967) *J. Biol. Chem.* 242, 3998-4006.
- Huttner, W.B., DeGennaro, L.J. and Greengard, P. (1981) *J. Biol. Chem.* 256, 1482-1488.
- Ingebritsen, T.S. and Cohen, P. (1983) *Science* 221, 331-338.
- Johnson, R.W. and Kenney, F.T. (1973) *J. Biol. Chem.* 248, 4528-4531.
- Johnson, R.A. and Walseth, T.F. (1979) *Adv. Cyclic Nucleotide Res.* 10, 135-167.
- Kenney, F.T. (1959) *J. Biol. Chem.* 234, 2707-2712.
- Kenney, F.T. (1962) *J. Biol. Chem.* 237, 1605-1609.
- Krebs, E.G. and Beavo, J.A. (1979) *Ann. Rev. Biochem.* 48, 923-959.
- La Du, B.N. and Gjessing, L.R. (1978) in *The Metabolic Basis of Inherited Disease* (Stanbury, J.B., Wyngaarden, J.B. and Fredrickson, D.S., Eds.) pp. 256-278.
- Laemmli, U.K. (1970) *Nature* 227, 680-685.
- Lee, K.-L. and Nickol, J.M. (1974) *J. Biol. Chem.* 249, 6024-6026.
- Lewis, E.J., Calie, P. and Wicks, W.D. (1982) *Proc. Natl. Acad. Sci. USA* 79, 5778-5782.
- Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265-275.
- Maizel, J.V. (1971) *Methods in Virology* 5, 179-246.
- McPherson, J.M., Whitehouse, S. and Walsh, D.A. (1979) *Biochemistry* 18, 4835-4844.
- Muneyama, K., Bauer, R.J., Shuman, D.A., Robbins, R.K. and Simon, L.N. (1971) *Biochemistry* 10, 2390-2395.

- Nestler, E.J. and Greengard, P. (1983) *Nature* 305, 583-588.
- Pierce, M.W., Palmer, J.L., Keutmann, H.T. and Avruch, J. (1981) *J. Biol. Chem.* 256, 8867-8870.
- Ramakrishna, S. and Benjamin, W.B. (1981) in *Protein Phosphorylation* (Rosen, O.M. and Krebs, E.G.) Cold Spring Harbor Conferences on Cell Proliferation Vol. 8, pp 747-758.
- Roach, P.J. (1981) in *Current Topics in Cellular Regulation* (Horecker, B.L. and Stadtman, E.R., Eds) Vol. 20, pp 45-105.
- Roper, M.D. and Wicks, W.D. (1978) *Proc. Natl. Acad. Sci. USA* 75, 140-144.
- Stellwagen, R.H. and Kohli, K.K. (1981) *Biochem. Biophys. Res. Commun.* 102, 1209-1215.
- Valeriote, F.A., Auricchio, F., Tomkins, G.M. and Riley, D. (1969) *J. Biol. Chem.* 244, 3618-3624.
- van den Berg, G.B., van Berkel, T.J.C. and Koster, J.F. (1980) *Eur. J. Biochem.* 113, 131-140.
- Wicks, W.D. (1969) *J. Biol. Chem.* 244, 3941-3950.
- Wicks, W.D. (1974) *Adv. Cyclic Nucleotide Res.* 4, 335-438.
- Wicks, W.D., Koontz, J. and Wagner, K. (1975) *J. Cyclic Nucleotide Res.* 4, 133-122.
- Wicks, W.D. and Su, J.-L. (1978) *J. Cyclic Nucleotide Res.* 4, 113-122.
- Wicks, W.D., van Wijk, R. and McKibbin, J.B. (1973) *Adv. Enz. Regul.* 11, 117-135.
- Wimalasena, J. and Wicks, W.D. (1979) *Mol. Pharm.* 6, 449-461.
- Wurtman, R.J., Larin, F., Mostafapour, S. and Fernstrom, J.D. (1974) *Science*, 185, 183-184.

VITA

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