To the Graduate council :

I am submitting herewith a thesis written by Joohong Park entitled "Pyrroloquinoline Quinone (Coenzyme PQQ) and the Oxidation of SH residues in proteins." 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.

Jorge E. Churchich, Major Professor

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and recommend its acceptance:

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PYRROLOQUINOLINE QUINONE (COENZYME PQQ) AND THE OXIDATION OF SH RESIDUES IN PROTEINS

A Thesis

Presented for the

Master of Science

Degree

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Joohong Park

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ABSTRACT

PQQ catalyzes the oxidation of cysteamine, dithiothreitol(DTT), and reduced glutathione,respectively, at neutral pH. The time course of cysteamine oxidation was monitored by absorption spectroscopy using the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) titrating reagent. Under pseudo first order condition, a second order rate constant for the conversion of cysteamine to cystamine was determined.

PQQ facilitates the oxidation of SH residues critically connected with the catalytic activity of enzyme from mammal;i.e., 4-aminobutyrate aminotransferase. PQQ treated enzyme recover catalytic activity upon addition of thiol compounds; cysteamine, DTT, and reduced glutathione, as well as thioredoxin from bacteria.

In addition to these experiments, 4-aminobutyrate aminotransferase was partially purified from bacteria (E. coli) , which was treated with PQQ by the same manner. Inactivated 4-aminobutyrate aminotransferase recovers catalytic activity upon addition of thiol compounds and the rate of recovery is accelerated by the addition of thioredoxin.

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CHAPTER I

INTRODUCTION

Quinoproteins are widespread among procaryotes; and it is well established that pyrroloquinoline quinone (PQQ) is the coenzyme of several bacterial dehydrogenase (1) . Although concentrations of PQQ below the mM range have been determined in mammalian tissues using the redox cycling assay (2), the functional role played by this coenzyme in eucaryotes remains to be elucidated. Free PQQ catalyzes non-enzymatic reactions at moderate pH and temperature values. Thus, oxidative decarboxylation of some aromatic amino acids takes place in the presence of cetyltrymethyl ammonium bromide (CTAB) (3), whereas the oxidation of pyridoxamine-5-P to pyridoxal-5-P was observed in the absence of micelles (4).

The studies of non-enzymatic reactions might serve as models for corresponding coenzyme dependent catalyzed reactions; and they might provide some insight into the role of PQQ in eucaryotes. The purpose of the present work is to report that coenzyme PQQ catalyzes the oxidation of small molecular weight thiols and the oxidation of vicinal SH residues in proteins.

CHAPTER II

EXPERIMENTAL PROCEDURES

Purification of Enzymes

A. 4-aminobutyrate aminotransferase from pig brain.

4-aminobutyrate aminotransferase was purified from pig brain tissues by procedures in our laboratory(5). Unless otherwise specified, the purification steps were performed at 4 °C and all the buffers contained 0.1 mM EDTA, 1mM 2-mercaptoethanol, and no pyridoxal 5-P. A waring Blender was used to prepare a 25 % (w/v) homogenate of pig brain in a solution of 10 mM potassium phosphate (pH 7) containing 1 mM 2-oxoglutarate. The resulting homogenate was adjusted to pH 5.5 by the slow addition of 1 M acetic acid, heated to 50 °C for 5 min. and after cooling to 4 °C, centrifuged at 10,000 xg for 30 min. The precipitate was discarded and the supernatant was treated with $(NH_4)_2SO_4$. The pellet obtained at 40 to 75 % saturation was dissolved in 0.01 M sodium acetate (pH 5.7) buffer (Buffer I) and dialyzed overnight against several changes of the same buffer. The dialysate was centrifuged at 10,000 g for 30 minutes and pellet diacarded. The supernatant was applied at a CM-sephadex column(5 x 80 cm) equilibrated with buffer I. The column was washed with 500 ml of the equilibration buffer and the enzyme

was eluted with linear gradient made with Buffer I (500 ml) and an equal volumn of 0.3 M sodium acetate buffer (pH5.7). At the end of the gradient elution, the column was washed with 250 ml of 0.3 M sodium acetate buffer(pH 5.7) to ensure complete elution of the enzyme. The enzyme eluted at an acetate concentration of 0.28 M.

The active fractions were pooled and then protein solution dialyzed extensively against 0.01 M potassium phosphate, pH 7.4 (Buffer II). The enzyme was applied to a column (2.6 X 30 cm) of DEAE-Sephadex equilibrated in the same buffer(Buffer II). The column was washed with 100 ml of Buffer II and the enzyme was eluted by using a linear gradient made with Buffer II (250 ml) and an equal volumn of 0.2 M potassium phosphate (pH 7.4). The enzyme was eluted at a phosphate concentration of 0.05 M.

The active fractions were pooled and then dialyzed against 0.01 M potassium phosphate (pH 7.0, Buffer III) (EDTA was omitted) and applied to a hydroxyapatite column(0.9 x 20 cm) previously equilibrated with Buffer III. The column was washed with 50 ml of Buffer III and the enzyme was eluted by using a linear gradient made with Buffer III(50 ml) and an equal volumn of 0.35 M potassium phosphate (pH 7.0). The enzyme was eluted at a phosphate concentration of 0.28 M.

Fractions which contained aminotransferase activity were subjected to polyacrylamide gel electrophoresis. Those fractions

judged to be electrophoretically pure were pooled and dialyzed against 0.1 M potassium phosphate (pH 7.4) buffer, Impure fractions were pooled and dialyzed against Buffer III and subjected to a second hydroxyapatite column chromatography.

A scheme of the purification is included in Table I

The enzyme has a specific activity of 20 units/mg of protein and a molecular weight of 100,000. A unit of enzyme activity is defined as the amount of enzyme needed to provide 1 μmol of succinic semialdehyde per min. at 25 ℃.

B. 4-aminobutyrate aminotransferase from E. coli

4-aminobutyrate aminotransferase from E. coli was purified by a procedure similar to that developed for the purification of the enzyme from Pseudomonas. sp. F-126 (6). The washed cells were suspended in 0.01 M phosphate buffer(pH 7.5) containing 0.01 % β-mercaptoethanol and then subjected to sonic disruption. The supernatant solution obtained by centrifugation was employed as the cell-free extract. Nucleic acids were precipitated with 0.2 % protamine sulfate and removed by centrifugation at 15,000 xg for 30 min. The supernatant was treated with ammonium sulfate, and the precipitate obtained at 35 to 70 % saturation was dissolved in 0.01 M phosphate buffer (pH 7.5) and dialyzed against several changes of

TABLE 1

Purification of 4-Aminobutyrate Aminotransferase from Pig Brain

Treatment	Volume	Protein	Specific activity at 25 °C	yield
	ml	mg/ml	units/mg	%
Homogenate	12,000	40	0.005	100
Supernatant after 50°C	6,000	6.2	0.022	34
$(NH_4)_2SO_4$(40-75 %)	800	10.4	0.035	12
CM-Sephadex fraction (0.01-0.3 M acetate pH 5.7)	200	9.9	0.094	7.8
DEAE-Sephadex fraction (0.01-0.2 M phosphate pH 7.4)	50	3.4	0.8	5.7
Hydroxyapatite (0.01-0.35 M phosphate pH 7.0)	5	1	20	4.2

the same buffer.

It was then applied to a DEAE-sephadex equilibrated with the same buffer and washed with the same buffer. The enzyme is not retained by DEAE-sepadex. The active fractions were combined, and concentrated using an amicon concentrator (membrane, PM 30). The protein solution was applied to a sephadex G-200 column (3 x 80 cm) and eluted with 0.01 M phosphate buffer (pH7.5). The elution profile of the enzyme corresponded to a protein of molecular mass of 180 KDa when compared to standards of known molecular weight. The active fractions were combined and applied to an hydroxyapatite column equilibrated with 0.01 M phosphate buffer (pH7.5) and eluted with a linear gradient made up of 0.01 M phosphate buffer (100 ml) and 0.3 M phosphate buffer (100 ml). The enzyme is eluted at aconcentration of phosphate buffer of 30 mM (Fig.1). The active fractions were assayed for catalytic activity and their purity tested by SDS-polyacrylamide gel electrophoresis. The fraction of greater specific activity (5 units/mg of enzyme) was used for the reaction with PQQ.

Enzymatic assays

Two methods were used in the assay of 4-aminobutyrate aminotransferase activity.

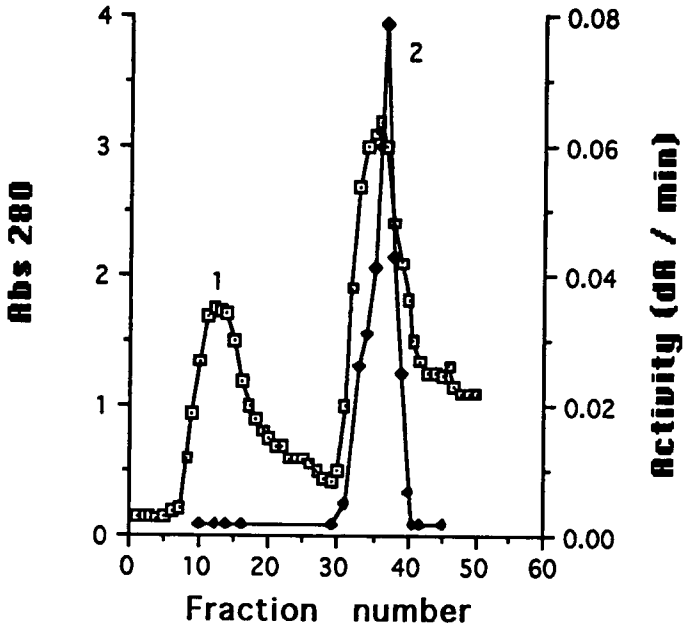

Figure 1. Chromatography of a fraction of 4-aminobutyrate aminotransferase from E.coli on hydroxyapatite. A linear gradient of potassium phosphate (low; 0.01 M, high;0.05 M) at pH 7.5 was produced. Fraction volumn about 1.5 ml, enzyme activity (1), OD_{280} (2)

Method I: This enzyme assay is based on fluorometric measurement of the condensation product of cyclohexane-1,3-dione with succinic semialdehyde(7). The reagent was a solution containing 0.25 g of cyclohexane-1,3-dione, 10 g ammonium acetate, 5 ml of glicial acetic acid in 100 ml of water. The substrate solution contains 20 mM 4-aminobutyrate and 10 mM 2-oxoglutarate adjusted pH 8.4 with NaOH. The substrate solution (5 ml) is incubated with the enzyme at 37 °C. Aliquots (0.5 ml) withdrawn from the incubation mixture at several time intervals were mixed with 0.4 ml of the reagent solution, heated for 16 minutes in a water bath at 60 °C , diluted to 3 ml by addition of water and the fluorescence intensity recorded at 460 nm with 365 nm excitation. A standard curve of succinic semialdehyde reacted with the cyclohexane-1.3-dione was run a parallel. This method permits the detection of succinic semialdehyde concentration lower than 0.1 μM without interference by the reagent cyclohexane-1,3-dione.

Method II: A coupled assay system consisting of two puryfied enzymes, i.e., 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase was used to study the catalytic conversion of 4-aminobutyrate into succinic semialdehyde.

Enzymatic assays were performed in 0.1 M sodium pyrophosphate (pH 8.4) containing 5 mM NAD^+, 20 mM 4-

aminobutyrate, and 10 mM 2-oxoglutarate. The progress of the reaction was followed by monitoring changes in absorbance at 340 nm due to reduction of transamination when the concentration of succinic semialdehyde dehydrogenase is at least five fold greater than the concentration of 4-aminobutyrate aminotransferase. Succinic semialdehyde dehydrogenase was puryfied from pig brain by a method already described (8).

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed according to the procedure of Davis(9). Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out at 25 °C as described by Lammli(10). The gel (7.5 % acrylamide) contained 0.1 % sodium dodecyl sulfate. Protein bands were detected by staining with Coomassie Blue dye for 1 hr and subsequently destained overnight in a solution containing 10 % methanol and 7 % acetic acid in water.

Spectroscopy

Fluorescence spectra were recorded in a fluorimeter equipped with two Bausch and Lomb monochrometers. The slits of the monochrometers were set to give a band width of 3 nm. Absorbance spectra were recorded in a shimadzu (UV-160) spectrophotometer.

The same instrument was used for kinetic studies.

Materials

Pig brains were obtained from Kodak company (Knoxville,TN) and bacteria (E. coli; Y1090 strain) were purchased from cloneteck. Thioredoxin from E. coli , purified from overproducing clones (11) , was a gift from Dr. Young Tae Kim. The concentration of purified thioredoxin was determined using an extinction coefficient E_{280} = 13.7 x 10^3 $M^{-1}Cm^{-1}$ and molecular weight of 11,660.

PQQ was purchased from Sigma; its purity was tested by HPLC following the procedure described in (12). Cysteamine, reduced glutathione, NAD^+, b-mercaptoethanol purchased from Sigma. DTNB was purchased from Aldrich chemical company. Sephadex G-25, Sephadex G-200, DEAE-Sephadex, CM-Sephadex were purchased from Pharmacia fine chemicals. Reagents for acrylamide gel electrophoresis including acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylenediamine and standards for protein molecular weight were purchased from Bethesda Research Laborotories.

All other chemicals were purchased from commercial sources and were of the highest purity available.

CHAPTER III

RESULTS

Reaction of PQQ with cysteamine

The extent of oxidation of cysteamine to cystamine was measured by monitoring the release of 2-nitro-5-mercaptobenzoate produced when 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) is allowed to react with aliquots of the reaction mixture containing PQQ (1.4×10^{-4} M) and cysteamine (2×10^{-3} M) at pH 7 at 25 °C (Table II).

The time course for a typical reaction under pseudo first order conditions is given in Fig 2. Plots of Kobs against varying concentrations of cysteamine yielded a second order rate constant $K_2 = 0.45\ M^{-1}\ Sec^{-1}$ for the disappearence of cysteamine.

The absorbtion spectrum of PQQ (max = 329 nm) is drastically changed following the addition of fourteen fold excess of cysteamine at pH 7 in 0.1 M phosphate buffer. An increase in the absorbtion band centered at 302 nm, together with a decrease in the absorbance covering the spectral range 330-500 nm are readily detected (Fig 3). This blue shift in the absorption spectrum of PQQ from 329 nm to 302 nm is a characteristic spectroscopic feature of reduced PQQ ($PQQH_2$) (12).

TABLE II

REACTION CATALYZED BY PQQ AT pH 7 [a)]

Substrate	Product	Rate (nmole / min.)	yield [b)]
cysteamine	cystamine	33	7.14
DTT	oxidized DTT	20	4.47
reduced glutathione	oxidized glutathione	9.4	2.04

a) 1 ml of the reaction mixture contained 0.14 mmol PQQ, 2 mmol of substrate in 0.1 M phosphate (pH 7.0) at 25 °C.

b) yield is expressed as the ratio of the concentration of product formed after 15 minutes of reaction to the concentration of PQQ.

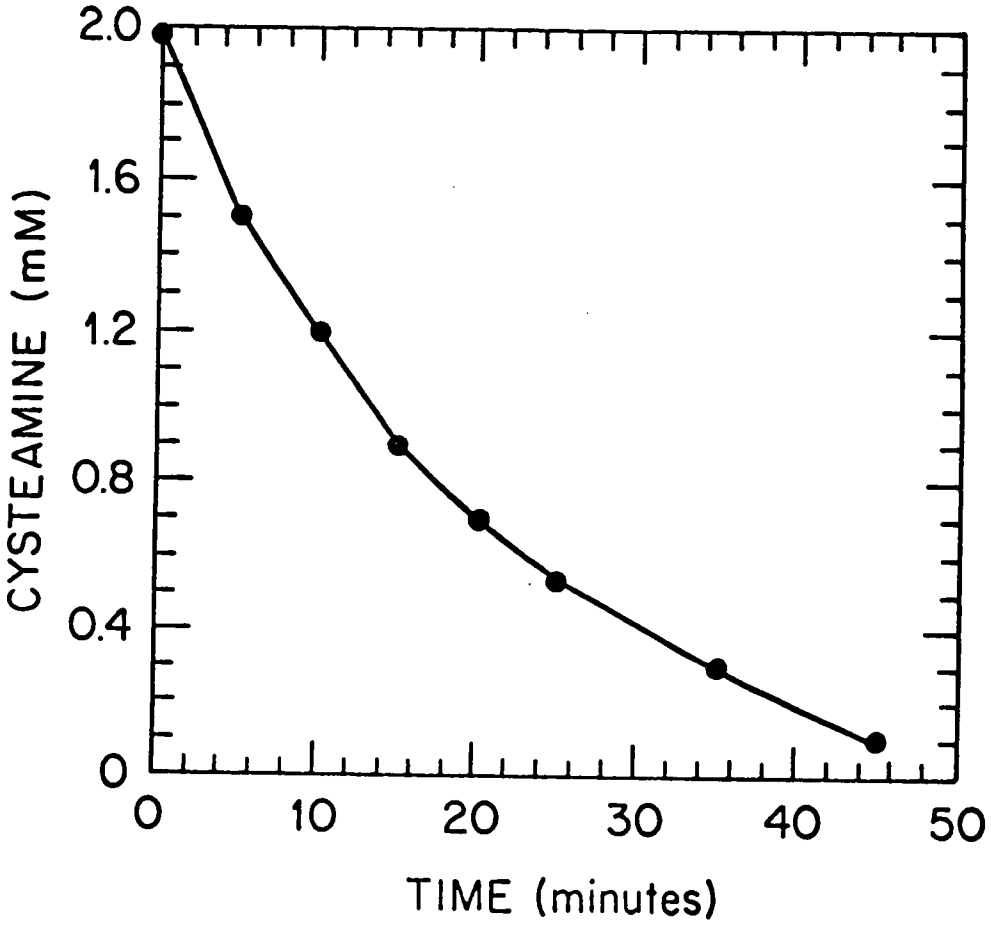

Figure 2. Time course of the reaction catalyzed by PQQ when cysteamine is used as substrate. Reaction of PQQ (1.4×10^{-4} M) with cysteamine (2×10^{-3} M) at pH 7 in 0.1 M phosphate buffer. Aliquots withdrawn from the reaction mixture at the indicated times were 10 fold diluted and titrated with DTNB (10^{-3} M) at pH 7.

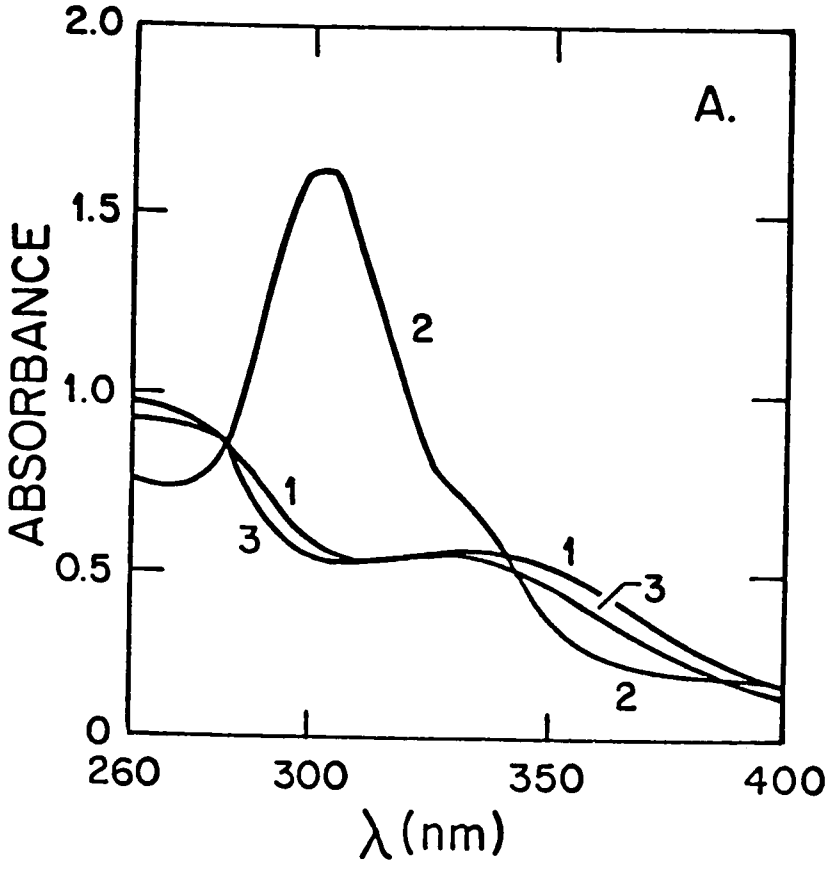

Figure 3. Absorption spectra of free PQQ and PQQ treated with cysteamine. PQQ (1.4 x 10^{-4} M) alone (1), PQQ (1.4 x 10^{-4} M) treated with cysteamine (2 x 10^{-3} M) at 25 °C for 2 minutes(2), for 3 hours 50 minutes(3).

Upon completion of the reaction the oxidation of $PQQH_2$ to PQQ leads to restoration of the original absorption spectrum (Fig. 3). The emission spectrum of PQQ is also perturbed by addition of cysteamine in the manner illustrated in Fig. 4. The characteristic emission band of PQQ centered at 480 nm is completely quenched following the addition of cysteamine. Once that cysteamine is oxidized to cystamine, the fluorescence properties of PQQ are restored. The same cycle, i.e., reduction and oxidation ofPQQ can be repeated several times provided cysteamine is added to the reaction mixture in the presence of oxygen.

Reaction of PQQ with 4-aminobutyrate aminotransferae from pig brain

4-aminobutyrate aminotransferase from pig brain is a dimeric protein made up of subunits of identical molecular weight(13). This mitochondrial enzyme catalyzes the reversible transamination of 4-aminobutyrate with the active site pyridoxal-5-P to yield succinic semialdehyde and pyridoxamine-5-P. Pyridoxal-5-P is reformed by transamination with α-ketoglutarate to yield glutamate and enzyme bound pyridoxal-5-P (7).

The oxidation of sulfhydryl groups in 4-aminobutyrate aminotransferase leads to the formation of disulfide bonds crosslinking the two subunits of the dimeric enzyme(14). Enzyme

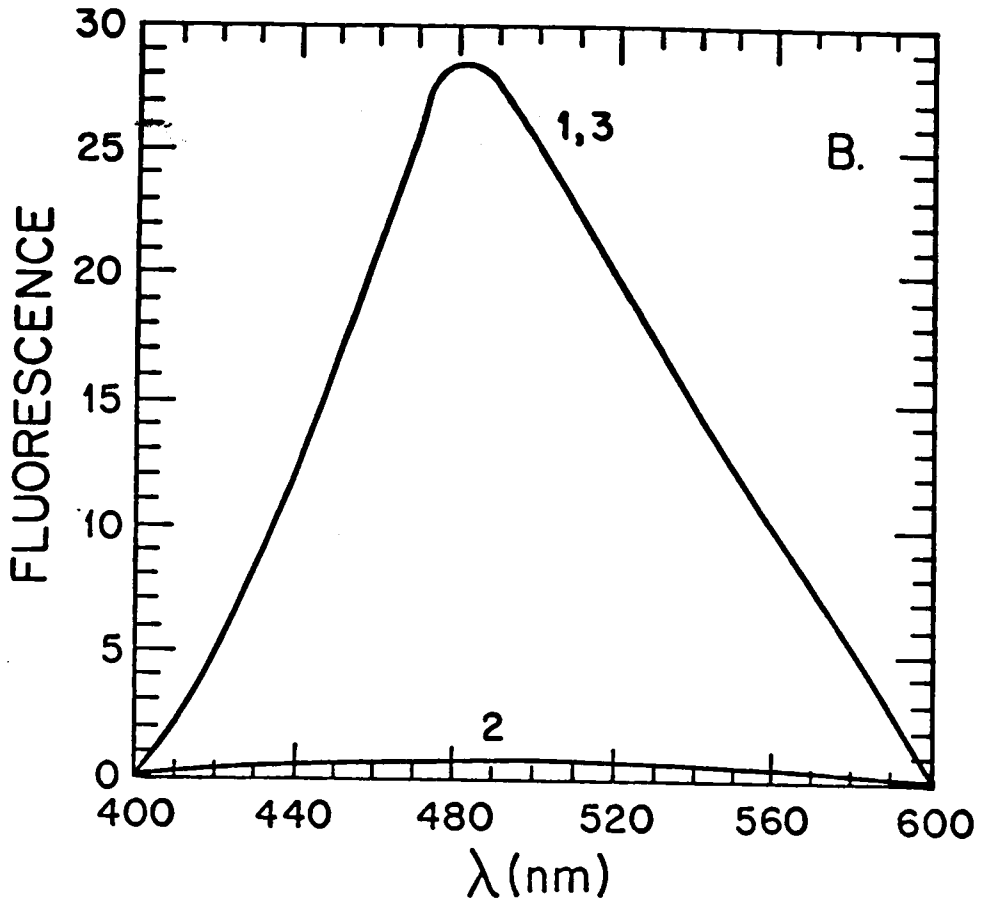

Figure 4. Emission spectra of free PQQ and PQQ treated with cysteamine. 1; free PQQ (1.4 x 10^{-4} M), 2; PQQ with cysteamine (2 x10^{-3} M) recorded after 1 minute of mixing, 3; PQQ (1.4 x 10^{-4} M) with cysteamine (2 x10^{-3} M) for 1 hour incubation at 25 °C. Excitation wavelength 380 nm.

species carrying disulfide bonds are inactive, but the recovery of catalytic activity is attained upon reduction of the disulfide bonds with either β-mercaptoethanol or reduced glutathione(15).

4-aminobutyrate aminotransferase is inactivated by preincubation with PQQ. Thus, the holoenzyme (10 μM) treated with PQQ (0.2 mM) in 0.1 M Tris-HCl buffer (pH 7.4) at 4 °C for 12 hours loses 90 % of its original activity. However, addition of cysteamine restores normal catalytic activity in the manner dipicted in Fig. 5, in the presence of 1 mM cysteamine, full recovery of normal catalytic activity is attained within 1 hour incubation at 25 °C.

To ascertain whether the loss of aminotransferase activity could be related to the modification of few cysteinyl residues, samples of modified enzyme were titrated with DTNB. The results of the titration experiments (Table III) indicated that 5.8 SH residues of the native enzyme were blocked by DTNB in the presence of 4 M Guanidinium-HCl, whereas 4.0 SH residues/dimer of modified enzyme have reacted with DTNB under similar experimental conditions. Hence, the loss of catalytic activity of the aminotransferase is related to the oxidation of approximately 2 SH residues/enzyme dimer.

Oxidation of SH residues catalysed by PQQ would produce either intersubunit cross-linking or intrasubunit cross-linking via

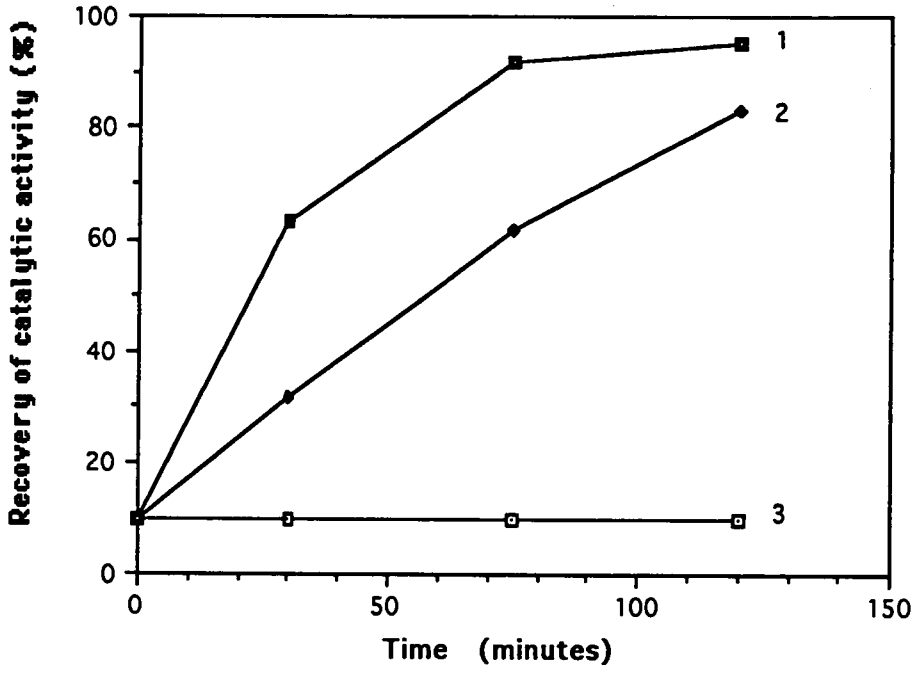

Figure 5. Reactivation of PQQ treated 4-aminobutyrate aminotransferase by cysteamine. 4-amonibutyrate aminotransferase (10 μM) preincubated with PQQ (0.2 mM) in Tris-HCl buffer (0.1 M, pH 7.4) at 4 °C for 12 hours. Recovery of the catalytic activity of 4-aminobutyrate aminotransferase is attained within 90 min. for incubation at 25 °C in addition of cysteamine 1; 1 mM cysteamine 2; 0.5 mM cysteamine 3; no cysteamine as a control

TABLE III

INACTIVATION OF 4-AMINOBUTYRATE AMINOTRANSFERASE BY PQQ [a)]

Sample	Activity (%)	SH residues mol/dimer c)
Aminotransferase	100	5.8
Aminotransferase + PQQ	5	4.0
Aminotransferase + PQQ + cysteamine b)	100	

a) 1 milliliter of the reaction mixture contained 1 mg of enzyme, 0.1 μmol PQQ in 0.1 M Tris-HCl (pH 7.4) incubated 12 hours at 4 °C.

b) 10 μM of the reaction mixture was two fold diluted with 1 mM cysteamine in 0.1 M Tris -HCl (pH 7.4), preincubated for 1 hour at 25 °C and assayed for enzymatic activity.

c) Titrated with DTNB in the presence of 4 M Guanidinium-HCl. The molecular weight of the dimer is 100,000.

disulfide bonds. If intersubunit cross-linking has taken place in the modified enzyme, then oligomeric species produced would be of molecular weight higher than 50,000 (monomer), and they would be detected by sodium dodecylsulfate-polyacrylamide gel electrophoresis. If, on the other hand, intrasubunit cross-linking via disulfide bonds is the result of the oxidation catalyzed by PQQ, then modified monomers would be expected to display the same electrophoretic mobility as monomeric species of 4-aminobutyrate aminotransferase. The electrophoretic patterns included in Figure 6 show the presence of monomeric species when the aminotransferase is inactivated by PQQ, suggesting formation of intrasubunit cross-linking via a disulfide bond.

Attempts to detect the reaction of specific amino acid residues of the enzyme with PQQ by means of spectroscopic methods were unsuccesful. Indeed, the modified protein displayed an absoption spectrum indistingushable from that of free PQQ over a wide spectral range : 310-450 nm.

Effect of thioredoxin on PQQ treated 4-aminobutyrate aminotransferase from pig brain

Thioredoxin is a small, ubiquitous protein with two redox-active half-cystine residues in an exposed active center, having two forms ; reduced form (thioredoxin-$(SH)_2$) and oxidized form

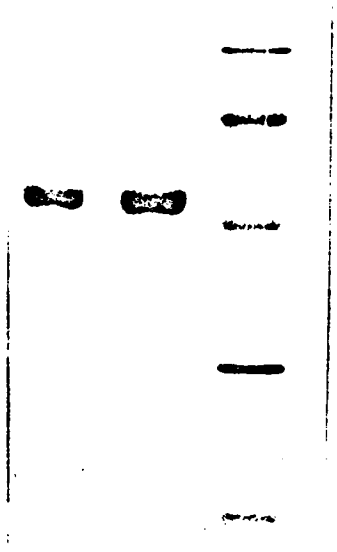

Figure 6. Sodium dodecyl sulfate polyacrylamide gel electrohoresis(7.5 %) of native 4-aminobutyrate aminotransferase and PQQ treated 4-aminobutyrate aminotransferase. lane 1; 4-aminobutyrate aminotransferase (20 mM), lane 2; 4-aminobutyrate aminotransferase (20 mM) treated with PQQ (1.4×10^{-4} M), lane 3; known molecular weight standard (rabbit muscle phosphorylase B(97 KDa), Bovine serum albumin (66 KDa), Ovalbumin (45 KDa), Carbonic anhydrase (31 KDa), Soybean trypsin inhibitor (21 KDa))

(thioredoxin-S 2). Thioredoxin participates in redox reactions through the reversible oxidation of its active center dithiol, to a disulfide, and catalyzes dithiol-disulfide exchange reactions(16).

Thioredoxin is present in many different procaryotes and eucaryotes and appears to be truly ubiquitous in all living cells. Bacteria, yeast, plant, and animal cells all contain thioredoxins with molecular weight around 12,000 that have evolved from a common ancester(17).

Thioredoxin is widely distributed in organs and subcellular fractions (e.g., nuclei, microsomes , membranes, and mitochondria) in mammalian cells(18).

Since the addition of cysteamine restores normal catalytic activity from the PQQ treated 4-aminobutyrate aminotransferase, it might be possible to use thioredoxin as a reducing agent for the same effect.

To test this possibility, thioredoxin was prepared by following manner. Thioredoxin treated with DTT (10^{-2} M) and passed through a sephadex G-25 column, equilibrated with 0.1 M phosphate buffer (pH5.8) is stable in the reduced state as long asthe value of pH is below 7.

The time course for reactivation of catalytic activity upon addition of thioredoxin was shown in Fig.7. About 50 % of recovery in catalytic activity was obtained with incbation for 30 minutes. In

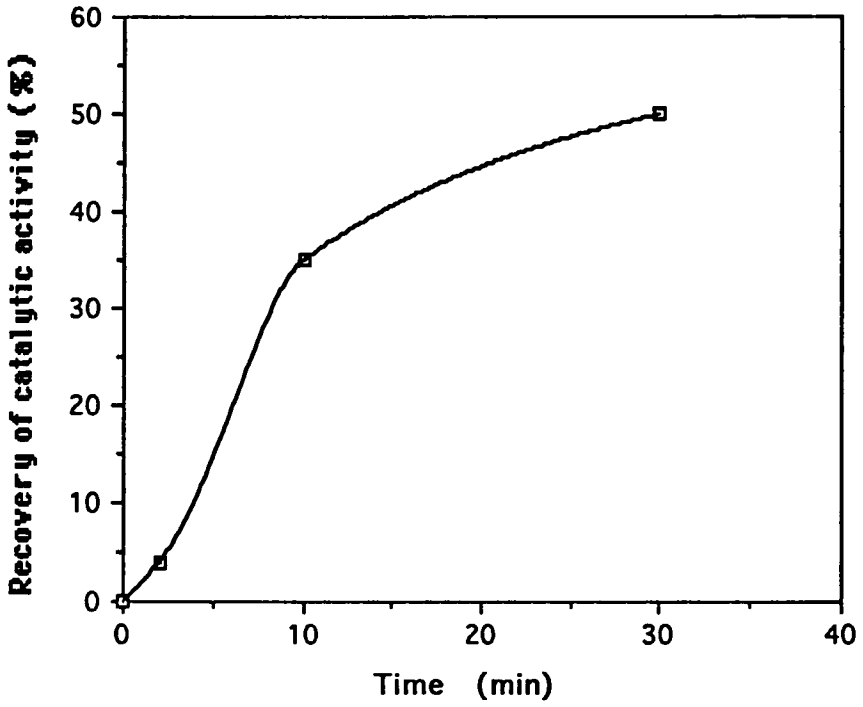

Figure 7. Time course of reactivation of PQQ treated 4-aminobutyrate aminotransferase by thioredoxin. 4-aminobutyrate aminotransferase was treated with PQQ (6x 10^{-4}) at pH 7 in 0.1 M phosphate buffer for 1 hour at 25 °C. The samples were dialyzed same buffer to remove excess of PQQ. Inactivated 4-aminobutyrate aminotransferase (10 μM) was incubated with reduced thioredoxin (10 μM) for each time interval and then measure the catalytic activity.

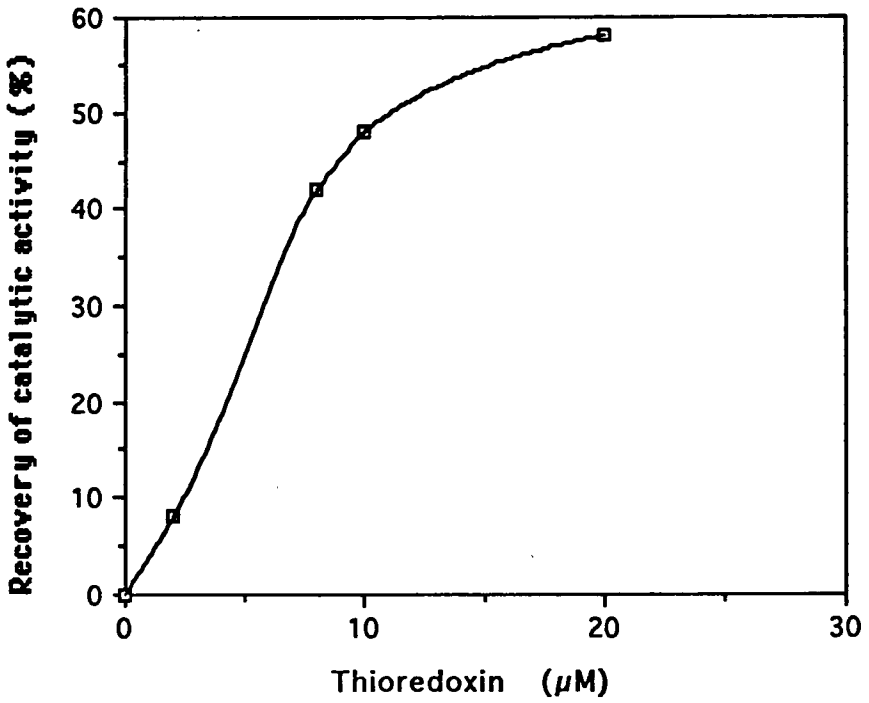

Figure 8. reactivation of PQQ treated 4-aminobutyrate aminotransferase by increased concentration of thioredoxin. 4-aminobutyrate aminotransferase was treated with PQQ (6x 10^{-4}) at pH 7 in 0.1 M phosphate buffer for 1 hour at 25 °C. The samples were dialyzed same buffer to remove excess of PQQ. Inactivated 4-aminobutyrate aminotransferase (10 μM) was incubated with different concentration of reduced thioredoxin for 30 minutes and then measure the catalytic activity.

addition to this experiment, the recovery of catalytic activity was determined with the increasing the concentration of thioredoxin (Fig. 8). These results indicates that reduced thiotrdoxin is able to catalyze the reduction of SH groups of enzymes.

Effect of thiotredoxin on PQQ treated 4-aminobutyrate aminotransferase from E. coli

Purified 4-aminobutyrate aminotransferase from E. coli, dialyzed against 0.1 M phosphate buffer (pH 7.5) to remove β-mercaptoethanol, was preincubated with PQQ and its catalytic activity measured using the coupled assay system. After addition of PQQ, and incubated at 25 °C for 1 hour, and dialyzed against 0.1 M phosphate buffer (pH 7.5) to remove free PQQ. The modified enzyme loses the original catalytic activity completely (Table IV).

Reactivation of the aminotransferase treated with PQQ is attained by addition of DTT in the manner depicted in Fig. 9. In the course of this reactivation process, an inactive form (oxidized enzyme) is converted into an active form (reduced enzyme) which catalyzes the transamination of 4-aminobutyrate.

Similar to the effect of thioredoxin on 4-aminobutyrate aminotransferase from pig brain, the addition of thioredoxin to the assay mixture increases the rate of reactivation as depicted in Fig. 10. This observation is interpreted to mean that the following

reaction take place at pH 8.5.

$$\text{Thioredoxin-S}_2 + \text{HS-R} \quad <-> \quad \text{Thioredoxin-(SH)}_2 + \text{R-S-S-R}$$

$$\text{E-S}_2 + \text{Thioredoxin-(SH)}_2 <-> \text{E-(SH)}_2 + \text{Thioredoxin-S}_2$$

The critical disulfide bond of thioredoxin formed between cys and cys is reduced by dithiothreitol(R-SH) ; then reduced thioredoxin acts on the oxidized enzyme to generate catalytically active species.

TABLE IV

INACTIVATION OF 4-AMINOBUTYRATE AMINOTRANSFERASE FROM E. coli BY PQQ AND CYSTAMINE a)

Sample	Activity (%)
Aminotransferase	100
Aminotransferase + PQQ	5
Aminotransferase + PQQ + DTT b)	100
Aminotransferase + Cystamine	1
Aminotransferase + Cystamine + DTT b)	100

a) 1 ml of the reaction mixture contained mg of enzyme, and 6 x 10^{-4} M PQQ, 10^{-2} M cystamine each, in 0.1 M phosphate buffer (pH 7.0) incubated 1 hour at 25 ℃.

b) 50 μl of the reaction mixture was taken and directly add into assay mixture containing 2 mM DTT.

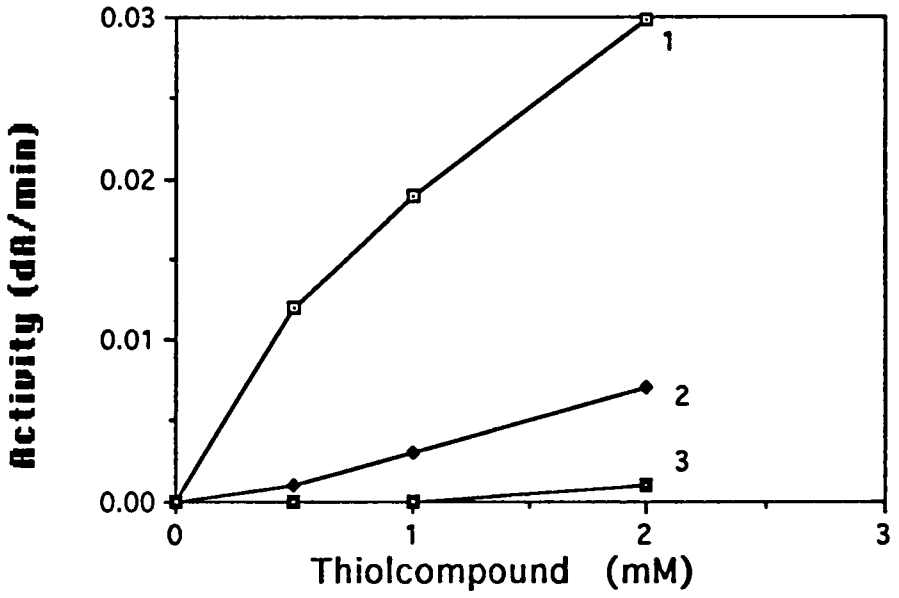

Figure 9. Reactivation of the PQQ treated 4-aminobutyrate aminotransferase from E. coli by addition of thiol compounds. 4-aminobutyrate aminotransferase (18 μM) was preincubated with PQQ (6 x10^{-4} M) in potassium phosphate buffer (0.1 M, pH 7) at 25 °C for 1 hour and dialyzed against same buffer to remove free PQQ. take an aliquot (0.1 ml), directly add into assay mixyure containing 1; 5 mM DTT, 2; 5 mM Cysteamine 3; 5 mM reduced glutathione.

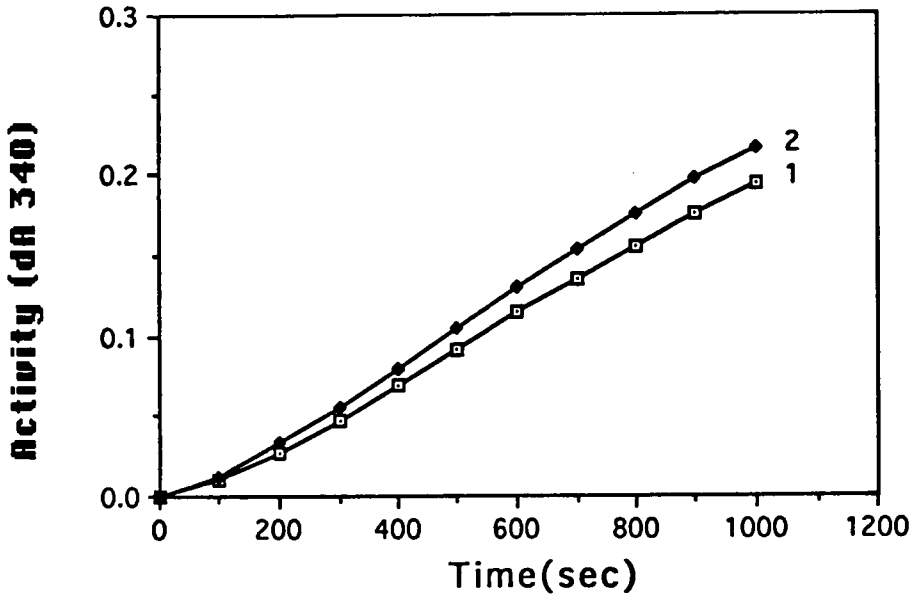

Figure 10. Effect on thioredoxin on reactivation of the PQQ treated 4-aminobutyrate aminotransferase from E. coli . 4-aminobutyrate aminotransferase (18 μM) was preincubated with PQQ (6 x10^{-4} M) in potassium phosphate buffer (0.1 M, pH 7) at 25 °C for 1 hour and dialyzed against same buffer to remove free PQQ. take an aliquot (0.1 ml), directly add into assay mixyure containing 1; 5 mM DTT, 2; 5 mM DTT + 5 μM thioredoxin.

CHAPTER IV

DISCUSSION

The present studies have shown that the oxidation of low molecular weight thiol compounds; i.e., cysteamine and reduced glutathione, is catalyzed by pyrroloquinoline quinone (PQQ) at neutral pH. Under identical experimental conditions of pH, PQQ and thiol concentrations, the oxidation of cysteamine proceeds three times faster than the oxidation of reduced glutathione.

Based on spectroscopic measurements and progressive decrease in the concentration of the reduced thiol compound, it is postulated that PQQ is reduced by the thiol compound in the early stages of the reaction. In subsequent steps, $PQQH_2$ is oxidized by O_2 to generate PQQ. Thus, a cycleic process takes place with catalytic amounts of PQQ.

An interesting aspect of the present work is the finding that PQQ facilitates the oxidation of two enzymes; 4-aminobutyrate aminotransferase from pig brain, and from bacteria (E. coli). both PQQ treated enzymes recover catalytic activity upon addition of thiol compound and thioredoxin.

The reaction of PQQ with 4-aminobutyrate aminotransferase from pig brain brings about the oxidation of approximately 2 SH

residues/dimer with concomitant loss of catalytic activity. Both PQQ treated aminotransferase are reactivated by thiol compounds as well as thioredoxin engaged in the reduction of disulfide bonds. Hence the following mechanism is proposed for the reaction catalyzed by PQQ and effect of thioredoxin.

$$PQQ + E\text{-}(SH)_2 \rightarrow PQQH_2 + E\text{-}S_2$$

$$PQQH_2 + 1/2\ O_2 \rightarrow PQQ + H_2O$$

$$E\text{-}S_2 + 2\ SH\text{-}R \rightarrow E\text{-}(SH)_2 + R\text{-}S\text{-}S\text{-}R$$

$$E\text{-}S_2 + \text{Thioredoxin-}(SH)_2 \rightarrow E\text{-}(SH)_2 + \text{Thioredoxin-}S_2$$

$$HS\text{-}E\text{-}S\text{-}S\text{-}R + \text{Thioredoxin-}(SH)_2 \rightarrow E\text{-}(SH)_2 + \text{Thioredoxin-}S_2 + SH\text{-}R$$

where SH-R : cysteamine ($HSCH_2CH_2NH_2$)

One might ask if the inhibitory effect of PQQ has any <u>in vivo</u> significance, The concentration of PQQ in mammalian tissues has been determined using the redox cycling assay (10). Of special interest is the report that PQQ content is relatively high in cerebrospinal fluid and in certain region of the brain (μM range)(11). This concentration level is far below the concentration of PQQ (0.1 mM) required to bring about inactivation of the enzymes examined in the present work. furthermore, the concentrations of

reduced glutathione in mammalian cells is above the mM range, and its rate of oxidation as catalyzed by μM concentrations of PQQ would be extremely slow. This implies that there is enough glutathione in mammalian cells to reduce PQQ, and consequently prevent the oxidation of SH residues of proteins.

Although it seems unlikely that the inhibitory effect exerted by PQQ has any regulatory function in eucaryotes, the ability of PQQ to oxidize SH groups in proteins and to generate disulfide bonds under mild experimental conditions can be used to define the role plaayed by those SH groups in the stabilization of tertiary structures.

It is well established that procaryotes contain quinoproteins, which catalyze the oxidation of several compounds. Furthermore, it has been demonstrated that free PQQ restores the catalytic function of some quinoproteins ; i.e., glucose dehydrogenase. None of those enzymes have been tested for their ability to catalyze the oxidation of free thiol compounds;i.e., cysteamine and low molecular weight oxidoreductases; i.e., thioredoxin. If it is shown that oxidation of thiol compounds and thioredoxin takes place in the presence of quinoproteins, then fluctuations in the concentrations of reduced thiol compounds and reduced thioredoxin might influence the catalytic function of some enzymes.

Thus, <u>in vitro</u> studies presentrd in this work have

demonstrated that 4-aminobutyrate aminotransferase from bacteria loses its catalytic function upon oxidation of SH groups. The recovery of catalytic activity depends only on the presence of thiol compounds which are engaged in disulfide-thiol exchange reactions.

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