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A SPECTROPHOTOMETRIC ASSAY FOR THE ENZYME CATALYZED REACTION OF 4-NITROQUINOLINE 1-OXIDE WITH GLUTATHIONE

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ABSTRACT

4-Nitroquinoline 1-oxide (4NQO(is a toxic and carcinogenic compound that has been reported to be subject to conjugation with glutathione (GSH). This reaction may proceed non-enzymatically or be catalyzed by GSH transferases. The non-enzymatic rate for this reaction has been reported to be very high. The purposes of this investigation were to develop a spectrophotometric assay for the reaction of 4NQO with GSH and to determine whether the rate for the enzyme catalyzed reaction was significant relative to the non-enzymatic reaction. The absorbance spectrum of 4NQO in phosphate buffer exhibited a maximum at 365 nm. Reaction of 4NQO with GSH was accompanied by a shift to 353 nm and an absorbance increase which was maximal at 350 nm. The formation of product could be quantitated from the increase in absorbance at 350 nm, where the change in the millimolar extinction coefficient was 7.20 mM^{-1} cm^{-1}. Although the non-enzymatic reaction of 4NQO and GSH proceeded rapidly at or above pH 8, at physiological pH this reaction was largely enzyme dependent. In an assay system containing 0.1 mM 4NQO, 1 mM GSH, and 0.1 M potassium phosphate, at 25°C, the conjugation of 4NQO with GSH by mouse liver cytosol was optimal at pH 6.5 - 7.5. At pH 6.5 and 1 mM GSH, a GSH transferase purified from mouse liver catalyzed the reaction of 4NQO with GSH with a maximum velocity of 156 μmoles/min per mg of protein. The K_m for 4NQO was 35 μM. The high activity of liver cytosol in promoting the reaction of 4NQO with GSH and the high affinity of the purified GSH transferase for 4NQO suggest that enzymatic catalysis of this reaction may be of considerable significance in vivo.

INTRODUCTION

4-Nitroquinoline 1-oxide (4NQO) is a toxic and carcinogenic compound that has been shown to cause tumors of the lung, forestomach, glandular stomach, mouth, esophagus, liver and skin of rodents (Ito, 1981). Both enzymatic and non-enzymatic conjugations of 4NQO with glutathione (GSH) have been reported (Al-Kassab *et al.*, 1963; Chasseaud, 1979). This reaction, shown in Figure 1, has been described as an addition-elimination reaction resulting in the formation of a thioether (Chasseaud, 1979). Two types of assays for the rate of reaction of 4NQO and GSH have been described. Varnes and Biaglow (1979) noted that both an increase in absorbance at 354 nm and the appearance of a fluorescent band at 420 nm appeared to accompany the formation of a GSH-quinoline 1-oxide conjugate. Changes in fluorescence in the 420 nm to 3000 nm range were used to quantitate

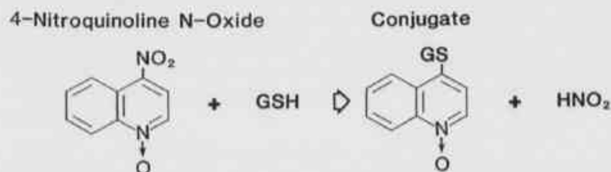

Figure 1. The conjugation of 4NQO with GSH.

the reaction of GSH and 4NQO. The absorbance increase at 354 nm was consistently observed, whereas the fluorescence at 420 nm was in some cases not detected. A causal relationship between the formation of product and the appearance of fluorescence at 420 nm appears not to have been established. Al-Kassab *et al.* (1963) measured the reaction of 4NQO with GSH by quantitation of the nitrite released. The non-enzymatic reaction was very rapid under the assay conditions used (pH 8.0, 6 mM GSH), and this imposed severe limitations upon the ease and applicability of the assay procedure. Nevertheless, these investigators were successful in demonstrating catalysis of the reaction by rat liver cytosol. The GSH transferases, a family of cytosolic detoxication enzymes, catalyze the conjugation of numerous xenobiotics with GSH (Jakoby and Keen, 1977; Habig, 1983). However, it has not been clear whether these enzymes may have a significant role in promoting the reaction of 4NQO with GSH, in relation to the rate at which this reaction occurs non-enzymatically.

The present investigation had two purposes — to develop a quantitative spectrophotometric assay for the reaction of 4NQO with GSH, and to establish whether the rate for the enzyme-catalyzed reaction was significant relative to the non-enzymatic reaction.

MATERIALS AND METHODS

Aldrich Chemical Company supplied the 4NQO. Sigma Chemical Company supplied the GSH. Preparation of mouse liver cytosol and of a purified GSH transferase was performed by methods that have been described previously (Pearson *et al.*, 1983). The GSH transferase isozyme, GT-8.8b, yielded a single coincident peak of enzyme activity and protein on preparative isoelectric focusing and a single Coomassie blue staining band after electrophoresis in polyacrylamide gels under denaturing conditions. In those assays catalyzed with mouse liver cytosol, 2 μl of 10-fold diluted cytosol were used. This represents 9.5 μg of protein in the 1 ml assay system. Spectrophotometric measurements were made on a Beckman DU-6 spectrophotometer with kinetics accessory. Product formation was linear with time and with enzyme concentration under the assay conditions used in these studies. Protein concentrations were measured by the method of Lowry *et al.* (1951).

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RESULTS

The absorbance spectrum of 4NQO in phosphate buffer at pH 6.5 and 25 °C exhibited a maximum at 365 nm (Fig. 2). Addition of GSH (6mM) to 4NQO (0.1 mM) yielded an increase in absorbance and a shift in the maximum to 353 nm. No further change was seen after 150 minutes. In the presence of mouse liver cytosol, 1.0 mM GSH, and 0.1 mM 4NQO, the identical spectral changes were observed. Figure 2 shows the spectrum of 4NQO and the spectrum of the product after complete enzymatic reaction with GSH. The difference spectrum (Fig. 3) shows that the maximum change in absorbance occurred at 350 nm. Product formation could be quantitated from the change in the millimolar extinction coefficient ($\triangle E$) at 350 nm, which was 7.2 $mM^{-1}cm^{-1}$. The dependence of the enzymatic and non-enzymatic reaction rates on pH was examined at 25 °C in an assay system containing 1.0 mM GSH, 0.1 mM 4NQO, and 0.1 M potassium phosphate. The enzymatic reaction was catalyzed by mouse liver cytosol. Figure 4 shows how these reaction rates were affected as the pH was increased from 5 to 8. The

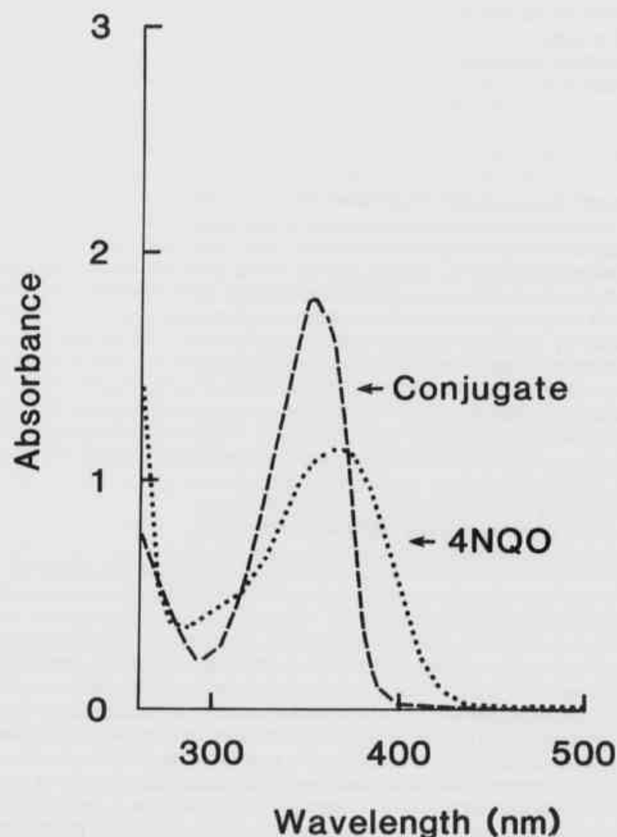

Figure 2. Absorbance spectra of 0.1 mM 4NQO and of the product formed by its complete enzymatic reaction with GSH (1 mM). Samples and blanks were in phosphate buffer, pH 6.5, at 25 °C and contained 0.2 μl of mouse liver cytosol.

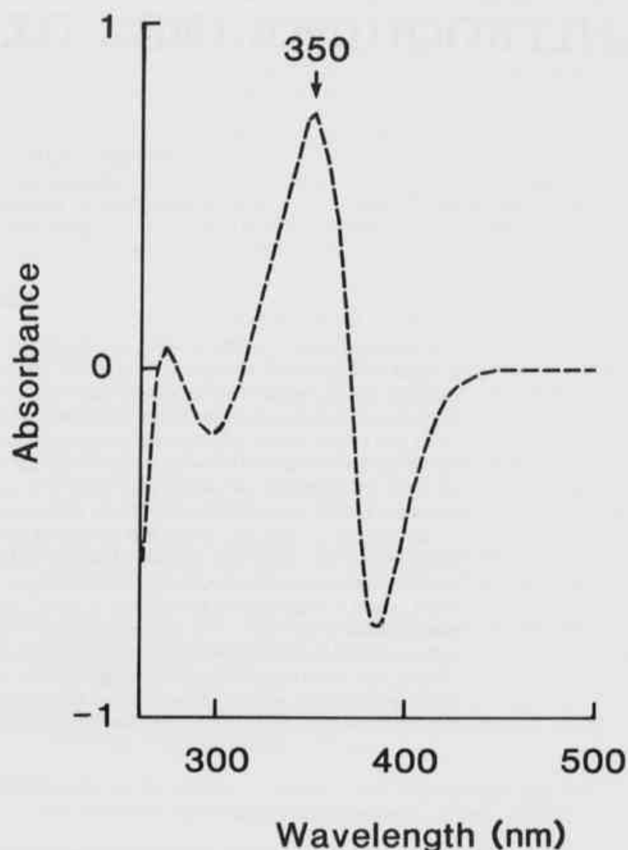

Figure 3. Difference spectrum of the reaction product of 4NQO and GSH, vs. 4NQO. The concentrations and conditions were as described in the legend to Fig. 2.

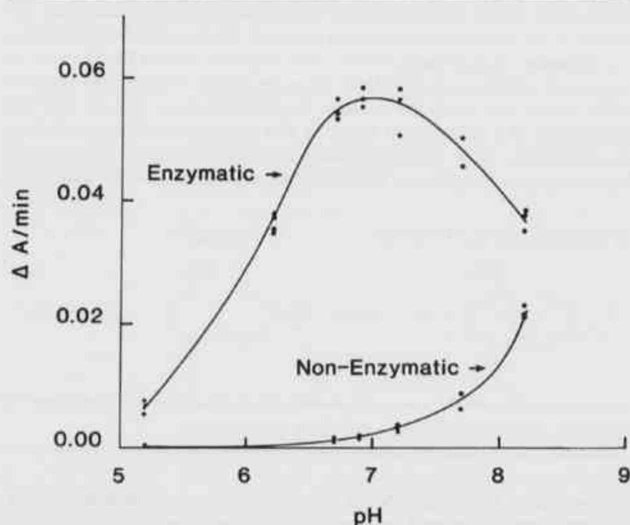

Figure 4. Enzymatic and non-enzymatic reactions of 0.1 mM 4NQO with GSH (1 mM) at 25 °C as a function of pH. The enzymatic reaction was catalyzed by 0.2 μl of mouse liver cytosol.

optimum enzymatic rate occurred between pH 6.5 and pH 7.5. The non-enzymatic rate rose with pH, increasing most rapidly above pH 7.7.

Some of the kinetic characteristics of the enzyme catalyzed reaction between 4NQO and GSH were examined, using a GSH transferase isozyme that had been purified to apparent homogeneity from mouse liver cytosol. The concentrations of 4NQO and GSH were optimized. The standard 1 ml assay system contained 0.1 M potassium phosphate, pH 6.5, 1.0 mM GSH, 0.05 mM 4NQO, and 0.06 μg of enzyme. The reaction was initiated by the addition of the 4NOQ in 10 μl of ethanol. The concentration of 4NQO was varied between 0.05 mM and 0.003 mM for the kinetics studies. The purified GSH transferase catalyzed the reaction of 4NQO and GSH with high efficiency. Kinetic analysis (Fig. 5) revealed that the enzyme had a K_m for 4NQO of 35 μM. The maximum velocity was 156 μmol/min per mg of GSH transferase.

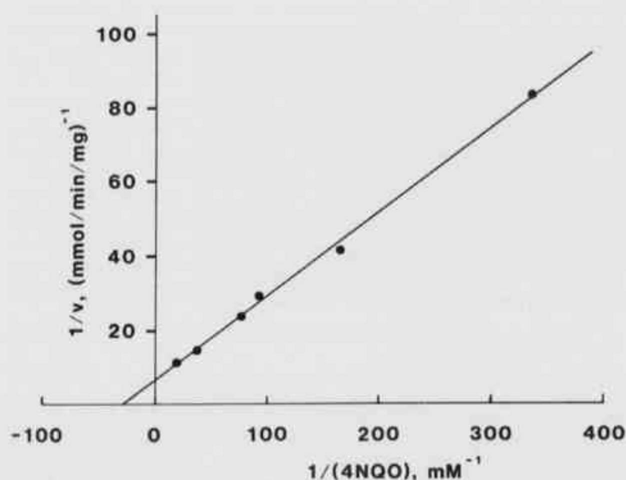

Figure 5. Double reciprocal plot of 4NQO concentration vs. initial velocity of the reaction of 4NQO with GSH (1 mM) in phosphate buffer at pH 6.5 and 25 °C. The reactions were catalyzed by a GSH transferse that had been purified from mouse liver cytosol.

DISCUSSION

The spectrophotometric assay described above is a rapid and convenient method for quantitating the reaction between 4NQO and GSH. The sensitivity was far greater than that obtained by measurement of nitrite released (Al-Kassab *et al.*, 1963), and product formation could be monitored continuously. Results obtained by application of this assay method showed that the non-enzymatic reaction of 4NQO with GSH increased with pH and became quite substantial at pH 8. However, in the most prevalent physiological pH range the role of glutathione transferases in promoting the reaction between 4NQO and GSH is clearly of much greater quantitative significance than the non-enzymatic reaction.

ACKNOWLEDGEMENT

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