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Glyoxalase I (EC 4.4.1.5) was purified from human red blood cells by a simplified method using S-hexylglutathione affinity chromatography with a modified concentration gradient of S-hexylglutathione for elution. The pure protein had a specific activity of 1830 U/mg of protein, where the overall yield was 9%. The pure protein had a molecular mass of 46,000 D, comprised of two subunits of 23,000 D each, and an isoelectric point value of 5.1. The $K_M$ value for methylglyoxal-glutathione hemithioacetal was $192.8 \pm 8 \mu M$ and the $k_{cat}$ value was $10.9 \pm 0.2 \times 10^4 \text{min}^{-1} (N=15)$. The glyoxalase I inhibitor S-p-bromobenzylglutathione had a $K_i$ value of $0.16 \pm 0.04 \mu M$ and S-p-nitrobenzoxycarbonylglutathione, previously thought to inhibit only glyoxalase II, also inhibited glyoxalase I with a $K_i$ value of $3.12 \pm 0.88 \mu M$. Reduced glutathione was a weak competitive inhibitor of glyoxalase I with a $K_i$ value of $18 \pm 8 \text{mM}$. The polyclonal antibodies were raised to the purified enzyme and were found to react specifically with glyoxalase I antigen by immunoblotting. This procedure gave a protein of high purity with simple low pressure chromatographic techniques with a moderate but adequate yield for small-scale preparations.

KEY WORDS: Glyoxalase I, methylglyoxal, glutathione, S-hexylglutathione affinity chromatography, immunoblotting.

I. INTRODUCTION

Glyoxalase I (EC 4.4.1.5) is a component of the glyoxalase system which catalyzes the conversion of methylglyoxal to D-lactate via the intermediate S-D-lactoylglutathione (Thornalley, 1990a). It is present in the cytosol of all cells. Glyoxalase I catalyzes the isomerization of the hemithioacetal, formed nonenzymatically from methylglyoxal and reduced glutathione, to S-D-lactoylglutathione.

$$\text{MeCOCHO} + \text{GSH} \rightarrow \text{MeCOCH(OH)-SG}$$

$$\text{glyoxalase I} \quad \rightarrow \quad \text{MeCH(OH)CO-SG}$$

The hemithioacetal is the molecular species which binds to the active site. The reaction is stereospecific, producing the D-lactoyl stereoisormer (Racker, 1954; Ekwall et al., 1973), and generally, with this and other glyoxal derivatives, the (R)-8-hydroxyacetyl-glutathione isomer is produced (Clelland and Thornalley, 1991). The reaction is thought to involve the formation of an ene-diolate intermediate formed from the (S)-hemithioacetal (Sellin et al., 1983; Douglas, 1987). However, both (R)- and (S)-forms of the hemithioacetal are consumed. Recent evidence from studies with (R)- and (S)-glutathiolaldehyde dehydrogenates suggests that both isomers of the hemithioacetate are accepted and are deprotonated in the initial mechanistic step by glyoxalase I, but there is stereospecific reprotonation of the ene-diol enzyme-bound intermediate (Landro et al., 1992).

The gene for human glyoxalase I, GLO, is on chromosome 6 (Bender and Greschik, 1976), close to
the major histocompatibility complex (Kompf et al., 1976). GLO lies between the centromere and HLA-DR: the meiotic distance between HLA-DR and GLO is ca. 6 cM (Bakker et al., 1979; Leach et al., 1986). There are three phenotypes, GLO 1-1, GLO 1-2, and GLO 2-2, representing the homozygous and heterozygous expression of a 2 allelic gene, GLO1 and GLO2 (Kompf et al., 1975). The distribution of glyoxalase I is widespread; indeed, it is thought to be ubiquitous, yet the role of this enzyme is uncertain (Thornalley, 1990a). However, there has recently been renewed interest in glyoxalase I, following studies showing its modification in processes of biological and clinical significance.

The activity of glyoxalase I was found to be modified in the cell growth cycle of human tumor cells in culture (Hooper et al., 1988a), and was increased during embryo maturation (Principato et al., 1982), during differentiation of liver following regeneration after hepatectomy (Principato et al., 1983), and during chemically induced differentiation of human leukemia (HL60 cells) in culture (Hooper et al., 1987, 1988b). Glyoxalase I activity was also increased during functional activation of human neutrophils (Thornalley and Bellavite, 1988; Thornalley et al., 1987). Recent research has also suggested there was a significant increase in glyoxalase I activity in red blood cells from insulin-dependent diabetic patients with complications (retinopathy) relative to uncomplicated patients (Thornalley et al., 1989). Further investigation is now required to characterize the cellular regulation and function of human glyoxalase I, particularly in tumor growth and the development of diabetic complications. To this end, we required a procedure for relatively small-scale (1-2 mg) isolation of human glyoxalase I from red blood cells.

The procedure reported by Schimandle and Vander Jagt (1979), using ethanol-chloroform precipitation and blue dextran chromatography, was performed but a poor yield was obtained. The alternative procedure of Mannervik et al. (1982) was described for a large-scale (20 L of packed red blood cells) and, as reported, gave poor yields when performed on a smaller scale. An improved method, taking advantage of the resolution of glyoxalase I from other proteins on a S-hexyl-glutathione affinity column by gradient elution recognized by investigators purifying glutathione S-transferases from red blood cells (Hayes, 1988), was developed.

In this report, we describe an improved and simplified method for the purification of human glyoxalase I from red blood cells. The purification procedure gave a homogeneous protein which was used to raise polyclonal antibodies. Studies of the inhibition of glyoxalase I with S-blocked glutathione derivatives revealed that S-p-bromobenzylglutathione was a potent inhibitor, and S-p-nitrobenzoxy carbonylglutathione—thought to selectively inhibit glyoxalase II (Bush and Norton, 1985)—was also a good competitive inhibitor.

2. MATERIALS AND METHODS

2.1. Materials

Outdated human blood was obtained from the Blood Transfusion Centre (Brentwood, Essex, U.K.) and was normally 3-6 weeks old. Methylglyoxal was synthesized from methylglyoxal dimethylacetal, purchased from Sigma Chem Co. Ltd. (Poole, Dorset, U.K.), by the published methods (McLellan and Thornalley, 1992). Reduced glutathione, free acid, and ammonium sulphate, grade III, were purchased from Sigma. S-Hexylglutathione was synthesized from reduced glutathione and hexyl iodide (Aldrich Chem. Co. Ltd., Poole, Dorset, U.K.), by the method of Vince et al. (1971) and was recrystallized twice from water/ethanol. Epoxy-activated Sepharose 6B was purchased from Pharmacia (Milton Keynes, U.K.) and was modified with S-hexylglutathione according to the manufacturer's instructions. Ultrafiltration membranes, PM10, were purchased from Amicon (Stonehouse, Gloucestershire, U.K.). Acrylamide, N,N'-methylene-bis-acrylamide, bromophenol blue, and N,N,N',N'-tetramethylethylenediamine (TEMED) were purchased from Sigma. Ampholine polyacrylamide plates (245 x 110 x 1 mm) in the pH range 4.0–6.5 were purchased from Pharmacia (Milton Keynes, U.K.).

2.2. Calibration of the Concentration of Stock Solutions of Methylglyoxal

The concentration of the stock aqueous solution of methylglyoxal was determined by endpoint assay involving conversion to S-o-lactoylglutathione with glyoxalase I and hydrolysis catalyzed by glyoxalase II (McLellan et al., 1992). An aliquot of methylglyoxal stock solution (diluted such that the final concentration was ~0.3 mM) was incubated with 2 mM reduced glutathione and 5 U of glyoxalase I (yeast; Sigma, type X) in 50 mM sodium phosphate buffer, pH 6.6 and 37°C; final volume 1 ml. The absorbance