結構與功能上探討人類葡萄糖六磷酸去氫酶上半胱氨基酸
Structural and functional studies of cysteine residues in human Glucose-6-phosphate dehydrogenase

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主持人：蔡淦仁　　私立中山醫學院醫事技術學系

一、中文摘要

紅血球細胞內的重要還原能力-NADPH 的產生，是經由六碳糖糖解分路上人類葡萄糖六磷酸去氫酶(G6PD)的酵素反應而來的。過去的研究中發現，(G6PD)的酵素缺乏，常常是由於 G6PD 基因突變所致，而導致酵素活性降低，進而造成疾病的產生。雖然在過去曾經利用過細菌的 G6PD 蛋白結晶，做了一些蛋白結構的研究。但由於人類與細菌 G6PD 序列上的明顯差異，因此將有必要更進一步瞭解人類 G6PD 上一些重要的氨基酸，及其所扮演的角色。其中在人類 G6PD 中發現有八個獨特的半胱氨基酸，在其他的 G6PD 所沒有的。在本研究中，我們將人類的 G6PD，利用定點突變的技術，製備具半胱氨基酸定點突變的 G6PD，以探討這八個半胱氨基酸的功能。我們初步的結果顯示，這八個半胱氨基酸可分為三大類:其中兩個半胱氨基酸，對於 G6PD 的活性是沒影響;三個半胱氨基酸，可能提供帶電的環境;而另外兩個半胱氨基酸，則可能產生雙硫鍵。

關鍵詞：G6PD、突變、半胱氨基酸

Abstract

Production of intracellular NADPH, an important reducing equivalent, in red cells is characterized by the enzyme reaction from glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49). Previous studies have revealed that the deficiency of the enzyme is largely associated with the gene mutation and resulted in the enzyme activity lost and subsequently disease developments. Although a previous bacterial crystalline study has provided a G6PD structure; however, due to the significant difference between bacterial and eukaryotic G6PD sequences, it will be necessary to reexamine the possible roles for some of these important residues in G6PDs. From amino acid sequence analysis of the deduced G6PD protein, eight cysteine residues are found unique to human G6PD, but not perfectly exist in its bacterial counterpart. Using site-directed mutagenesis techniques and enzymatic analysis, we investigated these eight cysteines in human G6PD. Our preliminary results have showed that these cysteines in human G6PD could be categorized into three different groups. Among them, two are not crucial for G6PD function, three are possibly providing an electrophilic environment for the enzyme activity, and the rest of the cysteine residues might be important for protein function by disulfide bond formation.

Keywords: Glucose-6-phosphate dehydrogenase; Site-directed Mutagenesis; Cysteine

二、緣由與目的

G6PD is one of important house keeping enzymes in biochemical reactions. G6PD catalyzes the oxidative reaction of D-glucose-6-phosphate to D-glucono-δ-lactone-6-phosphate in the hexose-monophosphate shunt. In G6PD enzyme reaction, NADPHs are generated to supply for the intracellular needs to repair oxidative damages especially in RBCs through the recycling reaction of oxidized glutathione (GSSG) to reduced glutathione (GSH) involved glutathione reductase [1] and in the presence of NADPH.

G6PD deficiency is the most commonly

found hereditary enzymopathy affects more than 200 millions people worldwide [2-4]. The incidence of the deficiency is rather high in Taiwan and therefore, it would be necessary to further understand this deficiency and its relationship to the disease development.

Chen *et al.* have cloned and sequenced the DNA of human G6PD gene [5] and it has been found that G6PD deficiency is largely associated with nucleotide mutations throughout the gene [6]. Some recent studies have focused on the structural and functional features of G6PD enzyme and its deficiency, and as a result of these researches, some functionally important domains have been revealed to the molecular levels [7-13]. Including the possible $NADP^+$ binding domain and the site for G6P binding have been proposed [7-8]. However, no crystalline structure for human G6PD protein is available, thus, the structural and functional relationship of G6PD has not been well characterized experimentally. Alternatively, using *Leuconostoc mesenteroides* G6PD structure as a model, which crystalline structure has been resolved recently [10], has been applied to disclose the structural information of human G6PD by amino acid sequence aligned to bacterial enzyme [11]. However, it is still very difficult to define the exact roles for each amino acids in human G6PDs, since the significance between human and bacterial G6PDs. Previously, we have investigated the roles of these arginine residues both structurally and functionally [13].

To further reveal the structural features of human G6PD protein, clearly define the roles of other important amino acids will be essential. Among them, eight cysteines are unique to human G6PD, but the number of cysteine residues are found less in other G6PDs. Interestingly, some bacteria contain less or even no cysteine in its G6PD. Therefore, it should be very important to delineate the roles of these cysteine residues in G6PD protein. As it has been recognized that the cysteine residues are sometime important for the protein structure as well as for the protein function. For example, these residues could provide thiol groups to participate the disulfide linkages of two distantly located cysteines and promote the formation of unique conformations/foldings. On the other hand, these residues might also perform a direct role in the enzyme function by contributing their thio- groups to the enzyme reaction. Previous studies of human erythrocytes have shown that the G6PD is inactivated by the mercuribenzoate treatment and accompanied by the release of one $NADP^+$ [15]. Indicating that there is at least a cysteine is involved in coenzyme binding in human G6PD [15]. Criss and McKerns [16] suggested a similar conclusion, and the roles for some cysteines have been established. However, none of these studies have provided clear-cut evidence for cysteine function in G6PD. On the contrast, Domagk *et al.* and Domschke *et al.* [17-18] provided evidence that cysteine is not required for yeast G6PD. Most interestingly, no cysteine is found in *L. mesenteroides* G6PD and it can be stated unequivocally that cysteine is no need for the enzyme activity [19]. In human G6PD, only one of the eight cysteines is linked to a G6PD deficiency, $G6PD^{Tomah}$[20]. Suggesting that cysteine might be either less important in G6PD or considerably significant, and thus any mutation at these cysteines resulted in the lethal consequences. If the latter statement is true, then no other cysteine mutations should be found among the newborns. Therefore, the roles for all cysteines in human G6PD activity will be worthy to review and to further characterize their functions in this protein.

Using site-directed mutagenesis method, we have generated cysteine mutants among the human G6PD and their enzyme activities were evaluated in this study. The possible roles for these cysteines were also discussed.

三、結果與討論

In this study, we have obtained an plasmid pYH1 and a parental plasmid pET5c for human G6PD protein expression in *E. coli* strain BL21(DE3) (from Dr. Chiu's laboratory). In the presence of IPTG, a significant

G6PD activity was measured in cell lysate prepared from *E. coli* strains harboring pYH1, but not from cells containing pET5c plasmid (data not shown). The *E. coli* expressed G6PD protein was also identified previously using SDS-PAGE and N-terminal portion of the protein was confirmed by amino acid sequencing (data not shown). Indicating that human G6PD is successfully expressed in this plasmid system in *E. coli*. We then use this *E. coli* expression system to explore the role of eight cysteines in human G6PD.

Using site-directed mutagenesis, we have prepared either serine or glycine substitution mutation at each cysteine residue. Cell lysates prepared from bacteria harboring either wild type or mutant G6PD plasmidwere used for G6PD activity assay as shown in Table 1.

Table 1. G6PD activity in wild type or cysteine mutant human G6PDs

	substitution	position	activity
Wild	no	no	100%
C13G	Glycine	13	40%
C13S	Serine	13	29%
C158G	Glycine	158	94%
C158S	Serine	158	95%
C232G	Glycine	232	30%
C232S	Serine	232	102%
C269G	Glycine	269	18%
C269S	Serine	269	94%
C294G	Glycine	294	58%
C294S	Serine	294	97%
C358G	Glycine	358	N.D.*
C358S	Serine	358	39%
C385G	Glycine	385	89%
C385S	Serine	385	96%
C446G	Glycine	446	35%
C446S	Serine	446	41%

*G6PD activity was undetectable

From our preliminary results obtained from this study, the functional roles for these eight cysteine residues can be categorized into three different groups:

1. Cysteines without functional role in human G6PD. Those cysteine mutants including C158G/C158S and C385G/C385S are belonged to this description. Since no G6PD activity changed were observed among these mutants either with an amino acid substitution of glycine or serine, it should be rationale that these cysteine residues at these two position play no functional role for G6PD activity.

2. Cysteines provide an electrophilic environment for G6PD activity. As those mutations lost their G6PD activities when cysteines were replaced by glycine, but not seen when serines were substituted, indicating that the cysteine residues at these positon might play a role in providing an electrophilic environment for G6PD activity. However, when cysteine mutated to a charged non-conserved glycine, the G6PD activity lost significantly. Based on the results, the Cysteines 232, 269, and 294 are fitted into this category.

3. Cysteines might play a role in disulfide bond formation. Other than those two different functions as mention above. There are three cysteine residues, which are crucial for the G6PD activity. Either changed to glycine or serine resulted in the reduce of G6PD activity dramatically. These sites include cysteines at position 13, 358 and 446, which cysteines are indispensable in G6PD activity. There are two possible roles for these cysteines including 1) these cysteine residues might provide their thio- group for the G6PD activity, however, there is no evidence so far that SH molecules involved in any enzyme process in G6PD reaction; 2) these cysteine residues might form disulfide bonds between each other or between cysteine residues from another G6PD protein molecule. As it has been demonstrated from previous structure study [32], G6PD might be function as a dimer form. Whether or not these cysteines provide the link between two G6PD molecules remain in doubt. However, it is also possibly that these cysteine residues might form an intracellular disulfide bond formation, since it have been demonstrated that the G6PD function is lost after the reducing agent treatment [15]. Therefore, it will be reasonable to suggest that the disulfide

bond(s) existed in G6PD and subsequently provide a correct folding for the protein.

In this study, we have shown that some cysteine residues we studied in the G6PD protein are important for enzyme activity. These cysteines are possibly conserved in human G6PD and for the enzyme activity. However, the exact role for all these cysteine residues remain to be answered. Especially the enzyme kinetics could provide only a portion of the story for G6PD protein structure, on the other hand, more biophysical data of G6PD structure will be needed to build up a more precise structural model for human G6PD protein. In order to achieve this goal, our laboratory is currently modified the previous expression and purification methods as those described above. And it will be expected that human G6PD protein could be prepared in a large amount and a human G6PD crystalline structure will be available in the near future.

四、計畫成果自評

Results shown in this report will be written in manuscript and submitted for publication if we can modify our protein production and re-examine the enzyme activity provided in this report. Due to the difficulty for obtaining a crystalline structure for G6PD protein, this research is thus significant for structural and functional studies of this enzyme, especially to those cysteine residues found only in humanG6PD. Although it is not conclusive enough to draw a precise picture for G6PD protein, however, the results shown in here provide an additional information to further understand those clinical complications related to those cysteines. Future studies of the G6PD will be necessary to achieve a crystalline structure to interpret the issues addressed in this report and it will be continuously undertaken in this laboratory.

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