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FOR THE RECORD

Site-directed mutagenesis to facilitate X-ray structural studies of *Leuconostoc mesenteroides* glucose 6-phosphate dehydrogenase

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The high specificity of most dehydrogenases for either NAD or NADP reflects the different metabolic roles played by these two coenzymes and has resulted in the evolution of two classes of nicotinamide coenzyme binding site, each of which allows only the "correct" coenzyme to bind. There are, however, a few dehydrogenases in which choice between NAD and NADP depends on the physiological conditions. Glucose 6-phosphate dehydrogenase (G6PD) (E.C. 1.1.1.49) from *Leuconostoc mesenteroides* is one such enzyme (De Moss et al., 1953). Our investigation of this enzyme has been motivated by a desire to understand the mechanism whereby the enzyme selects NAD or NADP for catalysis and the protein structural basis of this dual coenzyme specificity.

Earlier work (summarized by Levy [1989]) demonstrated that the NAD- and NADP-linked reactions catalyzed by *L. mesenteroides* G6PD proceed via different kinetic mechanisms, and that this difference appears to be the basis upon which the enzyme selects the appropriate coenzyme. Kinetic and binding studies are compatible with the binding of both coenzymes to the same site on the enzyme, whereas other experimental evidence (Kurlandsky et al., 1988) supports the idea that NAD binding produces a major conformational change, which is not seen upon binding NADP. The conventionally extracted (commercial) enzyme was crystallized and structural studies initiated (Adams et al., 1983), but the crystals proved to be difficult to work with as they were particularly sensitive to radiation and the damage was anisotropic. Furthermore, all attempts to prepare reproducible heavy atom derivatives were unsuccessful—in part perhaps because the enzyme lacks cysteine residues. The recent successful cloning and sequencing of the gene encoding *L. mesenteroides* G6PD (Lee et al., 1991) and the construction of a suitable strain of *Escherichia coli* in which this gene is expressed and from which large amounts of the enzyme can easily be purified (Lee & Levy, 1992) prompted us to use site-directed mutagenesis to address this problem. In this paper, we describe the preparation, characterization and crystallization of six cysteine-containing mutants of *L. mesenteroides* G6PD and the successful preparation and crystallization of a heavy atom derivative of one of these.

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Discussion

Serine and glutamine residues were selected for mutation to cysteine by virtue of their location in hydrophilic regions of the sequence that were predicted not to be in defined secondary structure and were seen not to be highly conserved. Secondary structure prediction from the amino acid sequence (Lee et al., 1991) was made using the Leeds package of algorithms (Eliopoulos et al., 1982). Regions of sequence identity and homology were identified by comparisons of the amino acid sequence of *L. mesenteroides* G6PD with those of G6PDs from human (Persico et al., 1986), rat (Ho et al., 1988), *Drosophila* (Fouts et al., 1988), *Saccharomyces cerevisiae* (Nogae & Johnston, 1990; Thomas et al., 1991), *E. coli* (Rowley & Wolf, 1991), and *Zymomonas mobilis* (Barnell et al., 1990), using a multiple sequence alignment and prediction protocol (Russell et al., 1992).

The plasmid pLmz, containing the wild-type *L. mesenteroides* G6PD structural gene (Lee et al., 1991), or a mutated plasmid, was inserted into *E. coli* strain SU294, which contains a deletion of the G6PD structural gene,

Table 1. *Oligonucleotides for cysteine mutation*

Mutation	Oligonucleotide[a]
Q56C	AT GAC GAA TTC AAA TGT TTG GTT CGT GAT TC
S61C	A TTG GTT CGT GAT TGC ATT AAA GAT TTC AC
S132C	C AAA TAT CTT AAG TGT GAA GGC CTA CTA GC
S215C	T CAA GTA ACA TTG TGC GAA GTC TTG GGT GT
Q365C	CA GAA CAA GAA GCA TGT GAA GCT GTC TTG TC
S428C	GT GAT GGC TGT AAC TTC GC

[a] Changes from wild type are underlined.

as previously described (Lee & Levy, 1992). The six cysteine mutants were constructed using the Amersham oligonucleotide-directed in vitro mutagenesis system version 2 and the plasmids sequenced, on single-stranded M13 subclones, to confirm the presence of the desired mutation and the absence of other mutations in the gene. The oligonucleotides, synthesized at the Syracuse University DNA and Protein Core Facility, are listed in Table 1; the designations of the strains containing the wild-type and mutant G6PDs are given in Table 2. All bacterial stocks were maintained on Luria–Bertani (Sambrook et al., 1989) agar plates containing 50 mg/mL of ampicillin (LB-AMP) and transferred monthly. For enzyme isolations, 5 mL of the LB-AMP medium was inoculated with bacterial colonies from a freshly prepared stock culture and grown at 37 °C overnight with vigorous shaking. This culture was used to inoculate 250 mL of the medium and the suspension was shaken on a rotary shaker at 37 °C for 24 h. G6PDs were routinely prepared from four 250-mL cultures, using the method described previously (Lee & Levy, 1992); 1 mM dithiothreitol was included in the buffers used during the isolation and storage of the mutant enzymes.

Table 2. *Catalytic properties of wild-type and cysteine mutant enzymes*

E. coli strain	Enzyme	Specific activity (units/mg protein)[a]	Activity ratio: NAD/NADP[b]
–	Wild type[c]	256	1.82
SU295	Wild type[d]	715	2.16
SU296	Q56C	432	1.95
SU297	S61C	468	1.95
SU298	S132C	495	2.09
SU299	S215C	677	1.65
SU300	Q365C	531	1.78
SU301	S428C	569	2.54

[a] NADP-linked activity under standard assay conditions (see text). Protein concentrations were measured using the Bradford microassay procedure (Bradford, 1976).

[b] Under standard conditions (see text).

[c] Data for wild-type enzyme isolated from *L. mesenteroides* are from Olive and Levy (1967). The specific activity of the commercial enzyme ranges from 187 to 385 units/mg protein.

[d] Data for recombinant wild-type enzyme are from Lee and Levy (1992).

The mutant G6PDs synthesized by each of the newly constructed *E. coli* strains represented at least 15% of total cell protein and each could be purified in high yield (45–80%). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was done by the method of Laemmli (1970); the gels were silver-stained for proteins (Merril, 1990) and indicated that each G6PD was nearly homogeneous prior to crystallization. The NAD- and NADP-linked activities were measured at 25 °C by following the rate of NAD(P)H appearance at 340 nm. Assays were initiated by the addition of enzyme to 33 mM Tris-HCl at pH 7.6, containing either 1.6 mM glucose 6-phosphate and 114 μM $NADP^+$ or 1.06 mM glucose 6-phosphate and 21.2 mM NAD^+, neutralized to pH 7.0; these substrate and coenzyme concentrations are 20 times the respective K_m values for the wild-type *L. mesenteroides* G6PD (Levy et al., 1983). The specific activities and ratios of NAD- to NADP-linked activities, shown in Table 2, indicate that the six mutant G6PDs are similar to the wild-type *L. mesenteroides* G6PD isolated from *E. coli*. These activities are substantially higher than that of the enzyme originally isolated from *L. mesenteroides* (Olive & Levy, 1967; also shown); this may reflect microheterogeneity in the latter, as is observed in the commercial enzyme (Lee et al., 1991).

Crystals of all samples of recombinant enzyme have been grown from phosphate by the hanging drop procedure. The conditions giving optimal crystals (enzyme concentration 4–6 mg/mL; 3.0–3.2 M phosphate in well), although varying among mutants, are all comparable with those used for the commercial enzyme (enzyme concentration 3.5 mg/mL; 3.2 M phosphate in well) (Adams et al., 1983). The nucleation time varies from 1 to 4 weeks, and crystals are typically 1.2 × 0.2 mm. Wild-type enzyme crystallizes in the space group $P3_121$ ($P3_221$) with cell dimensions $a = b = 105.71$ (11) Å, $c = 224.31$ (15) Å. All mutant crystals grown so far are isomorphous with wild-type crystals and with each other, their a dimension varying from 105.47 (4) Å to 105.81 (12) Å and c dimension from 224.07 (6) Å to 224.64 (21) Å. Comparison with the cell dimensions of crystals grown from the commercial enzyme ($a = b = 105.8$ Å, $c = 225.1$ Å) indicates a significant shortening of the c axis for all recombinant enzymes.

X-ray diffraction data have been recorded using Cu Kα radiation. Data for the commercial enzyme and potential derivatives were originally routinely collected to 5 Å resolution on a Xentronics area detector; data to 3.5 Å for the native enzyme and some 5-Å data sets for crystals of recombinant enzymes have also been collected this way. Data sets are now routinely collected to 3.5 Å resolution on a MAR Research image plate with an additional collimator installed between the monochromator and the detector collimator to allow adequate resolution of reflections along the long c axis. A complete data set to 3.5 Å

may now be obtained from two or three crystal parts, as all recombinant enzyme crystals have a much better radiation damage profile than those grown from commercial enzyme. The native data set, collected on a Xentronics area detector to 3.5 Å resolution from the commercial enzyme, required three crystals and was 78% complete, containing 14,619 independent reflections to 3.51 Å. Temperature factors varied by 20 Å^2 over the data set and the anisotropy by 10 Å^2; the merging residual (R_M[1]) for 29,325 measurements of 10,488 reflections was 10.2% (9.0% for reflections with $I/\sigma I > 4$). A comparable data set from recombinant wild-type crystals used three crystals to collect a 98.8% complete 3.53 Å set (18,199 independent reflections) with temperature factors varying by 8 Å^2 and the anisotropy by 2 Å^2; R_M for 59,205 measurements of 16,709 reflections was 5.23% (4.73% for reflections with $I/\sigma I > 4$).

Heavy atom derivative preparation has been attempted both by co-crystallization and by soaking crystals of mutant enzyme in solutions containing heavy compounds. Because all mutants are stored in dithiothreitol, enzyme is dialyzed in buffer without dithiothreitol prior to addition of mercurial or gold compound. Co-crystallization experiments have used *p*-hydroxymercuribenzoate (PHMB) and potassium dicyanoaurate (I); soaking experiments utilized a range of mercurials soluble in 3.0 M phosphate at pH 5.8.

Data from attempted co-crystallizations of Q56C, S215C, and Q365C with PHMB under the same conditions have been collected to 3.5 Å resolution. All crystals were isomorphous with wild type, with mean fractional isomorphous differences varying from 12% to 16%. Difference Pattersons indicate substitution in S215C-PHMB (Fig. 1) but no substitution in the other mutants. The S215C-PHMB difference Patterson was solved using a peak search routine: there are two mercury atoms present per asymmetric unit, and the 14 peaks indicating self vectors and cross vectors are the highest nonorigin peaks in the difference Patterson. Heavy atom parameters after centric refinement are given in the legend to the figure: the two noncrystallographically related heavy atoms have slightly different occupancies and temperature factors, reflecting their different environments in the crystal. Phasing for all data gives a ratio of heavy atom scattering (f_H) to lack of closure error (E) of better than 4.0 inside 7 Å, falling to 1.13 at 3.5 Å.

Structure factors have been input to the Wang solvent flattening procedure (Wang, 1985), using stepwise phase extension in resolution shells. The resulting phase set will be used in a search for further derivatives and to determine the relationship between the two independent subunits of the enzyme within the crystallographic asymmetric

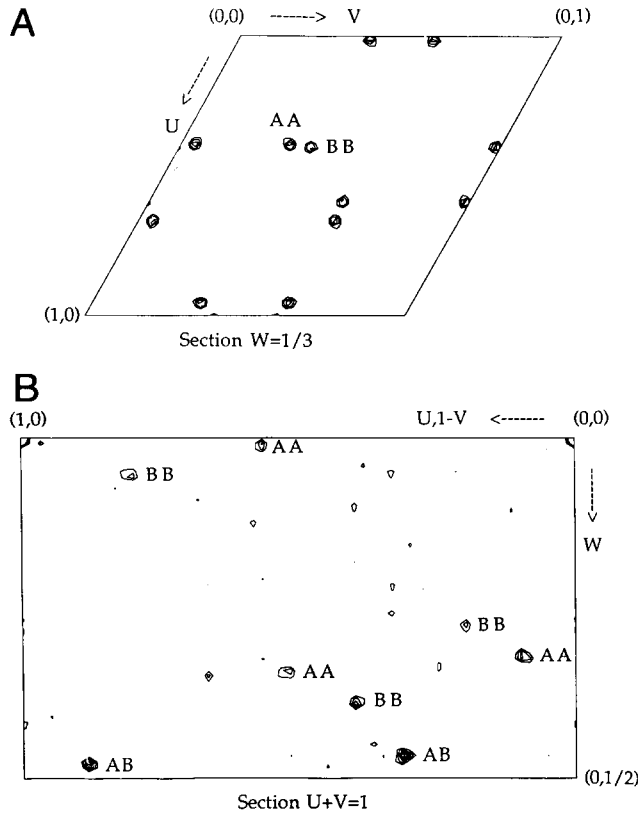

Fig. 1. Harker sections: W = 1/3 (**A**) and (U + V) = 1 (**B**) of the difference Patterson between S215C-Hg-G6PD and wild-type G6PD, contoured from 20 by 10 to 200 (A) and from 15 by 10 to 195 (B). The origin is 1,000. These sections contain the required eight self vectors and two of the six cross vectors for the two mercury sites in the asymmetric unit. The refined mercury parameters (occupancy Z on an arbitrary scale, temperature factor B, fractional coordinates x, y, z) are, for site A: $Z = 2.84$, $B = 18.5$ Å^2, $x = 0.4753$, $y = 0.5687$, $z = 0.3388$ and, for site B: $Z = 2.51$, $B = 27.0$ Å^2, $x = 0.6032$, $y = 0.8096$, $z = 0.8618$.

unit. Data will be extended to higher resolution. The solution of the crystal structure will use noncrystallographic symmetry averaging and further solvent flattening, as well as isomorphous replacement and anomalous scattering.

Previous success in engineering heavy atom binding sites by insertion of accessible cysteines into proteins such as U1 snRNP protein A (Nagai et al., 1990) suggests this may be a method of choice when a good expression system is available. Single amino acid mutants of *L. mesenteroides* G6PD were prepared in which cysteine replaced serine or glutamine residues. Because the mutants have normal activity, substitution is removed from the active site. The crystals prepared from these mutants are all isomorphous with the wild-type enzyme, suggesting that replacement of the chosen residues has disrupted neither the native structure nor the crystal packing.

For the solution of the structure, the recombinant enzymes have afforded advantages over the commercially prepared enzyme in two respects. First, the crystals are

[1] $R_M = (\Sigma_h \Sigma_{i=1,N} |I_{hi} - \langle I_h \rangle|)/(\Sigma_h N \times \langle I_h \rangle)$, where h is the reflection index and $\langle I_h \rangle$ is the mean of N equivalent measurements (I_{hi}) of intensity.

substantially more resistant to radiation damage. The second advantage is that heavy atom derivatives can be prepared from at least one of the mutant G6PDs. The inaccessibility of much of the enzyme surface when in the crystal was suggested by the lack of success in preparing heavy atom derivatives of the commercial enzyme; this has been reinforced by the repeated growth of underivatized crystals in co-crystallization experiments with the Q56C and Q365C mutants. Successful derivatization of the S215C mutant confirms that an accessible cysteine provides an excellent heavy atom binding site.

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