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Detection of L-lactate in polyethylene glycol solutions confirms the identity of the active-site ligand in a proline dehydrogenase structure

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Polyethylene glycol (PEG) is often used in protein crystallography as a low-ionic-strength precipitant for crystallization and as a cryoprotectant for low-temperature data collection. Prompted by the discovery of an apparent L-lactate molecule bound in the active site of the *Escherichia coli* PutA proline dehydrogenase domain crystal structure, the L-lactate concentration of several PEG solutions was measured. 50%(*w*/*v*) solutions of PEGs with molecular weight 3000, 4000 and 8000 contain millimolar levels of L-lactate. In contrast, L-lactate was not detected in solutions of PEG monomethyl ethers or PEG 3350. These results help to explain why L-lactate was present in the proline dehydrogenase domain crystal structure. This work also has implications for the crystallization of enzymes that bind L-lactate.

1. Introduction

Polyethylene glycol (PEG) was first used successfully as a precipitating agent for protein crystallization in the mid-1970s, when Ward and coworkers reported the structure of deoxyhemoglobin using crystals grown in PEG (Ward *et al.*, 1975). The next year, McPherson firmly established PEG as an important crystallization reagent by crystallizing 13 proteins using a variety of PEGs. He concluded that PEG might be the best initial trial reagent for crystal screening (McPherson, 1976). PEG monomethyl ether (PEGMME) was added to the protein-crystallization arsenal in 1994 (Brzozowski & Tolley, 1994). Today, PEG and PEGMME are the most commonly used precipitating agents for protein crystallization, based on their inclusion in about half of the reagents in Crystal Screen, Wizard and Index crystal screens. Therefore, it is of interest to know the identities and concentrations of contaminating species present in commercially available PEGs and PEGMMEs. For example, Ray and Puvathingal discovered that rabbit muscle phosphoglucomutase lost activity within a few hours after exposure to 3% PEG 400, which prompted them to develop a chromatographic procedure for removing contaminating aldehydes and peroxides from PEGs (Ray & Puvathingal, 1985).

Here, we present an analysis of L-lactate levels in PEG solutions commonly used in protein crystallization. This study was motivated by the discovery of an apparent L-lactate molecule bound in the active site of the *Escherichia coli* PutA proline dehydrogenase (PRODH) domain crystal structure (Lee *et al.*, 2003), despite the fact that lactate was not knowingly added to the protein solution used for crystallization (Nadaraia *et al.*, 2001). Since the crystals were grown in 24%(*w*/*v*) PEG 3000 and L-lactate had been detected in PEG 1000 previously (Pollegioni *et al.*, 2002), we suspected that PEG might be the source of L-lactate. Using a coupled enzymatic assay, we find that stock solutions of PEG 3000, 4000 and 8000 contain millimolar levels of L-lactate, while PEGMME 2000, PEGMME 5000 and PEG 3350 are apparently free of L-lactate. These results confirm the identity and source of the active-site ligand in the PRODH crystal structure. They also suggest that PEGMMEs and PEG 3350 might be useful reagents for crystallizing PRODH and other enzymes known to bind L-lactate.

2. Materials and methods

The procedure described by Noll (1983) was used to determine L-lactate levels in aqueous PEG solutions. This method is an end-point assay involving two enzymes: NAD-linked L-lactate dehydrogenase (LDH) and L-alanine aminotransferase (ALT). LDH catalyzes the oxidation of L-lactate to pyruvate, thereby reducing NAD^+ to NADH. ALT converts pyruvate and L-glutamate to L-alanine and 2-oxoglutarate. The ALT reaction is necessary to remove pyruvate from the system because the equilibrium of the LDH reaction favors the formation of lactate from pyruvate. The final NADH concentration, which is monitored by the absorbance at $\lambda = 339$ nm, is proportional to the L-lactate concentration in the sample. The detection limit of this method is 0.07 m*M* (Noll, 1983).

The assay protocol was used as described previously (Noll, 1983) except the concentrations of LDH, ALT and NAD^+ were increased fourfold, fourfold and fivefold, respectively, in

Table 1
L-Lactate concentrations of PEG-containing reagents.

BD = below detection limit.

Reagent	[PEG] [%(*w*/*v*)]	[L-Lactate] (m*M*)
PEG 3000 (Fluka 81227)	50	3.5 ± 0.2
PEG 4000 (Fluka 81240)	50	3.0 ± 0.2
PEG 4000 (Crystal Screen No. 6)	30	2.1
PEG 8000 (Fluka 81268)	50	2.3
PEG 8000 (Crystal Screen No. 28)	30	1.8
PEG 8000 (Wizard 1 No. 1)	20	1.2
PEG 8000 (Wizard 1 No. 17)	30	1.8
PEG 8000 (Wizard 1 No. 31)	20	1.1
PEG 3350 (Sigma P-4338)	50	BD
PEG 3350 (Index No. 45)	25	BD
PEGMME 2000 (Fluka 81321)	50	BD
PEGMME 2000 (Index No. 47)	28	BD
PEGMME 5000 (Fluka 81323)	50	BD
PEGMME 5000 (Index No. 46)	20	BD

order to decrease the overall reaction time (Noll, 1983). The increased concentrations of LDH, ALT and NAD^+ were within the recommended ranges (Noll, 1983). Rabbit muscle LDH and porcine heart ALT were purchased from Sigma (catalog Nos. L1254 and G9880) and used without further purification. The following reagents were purchased from Sigma: L-(+)-lactate (catalog No. L1750), NAD^+ (catalog No. N1511) and L-glutamic acid (catalog No. 49449). The assays were performed using a Cary 100 UV–visible spectrophotometer equipped with a 6 × 6 multi-cell transporter.

Several commercially available PEGs were tested for the presence of L-lactate. PEGs of various molecular weights were purchased from Fluka and Sigma (see Table 1 for catalog Nos.) and aqueous solutions were prepared by dissolving the PEGs in deionized water (18 MΩ) produced by a MilliQ Synthesis water-purification system. Selected reagents from Crystal Screen (Hampton Research), Index (Hampton Research) and Wizard 1 (Emerald Biostructures) crystals screens were also analyzed. The L-lactate concentrations reported in Table 1 for PEG 3000 (Fluka), PEG 4000 (Fluka) and PEG 3350 (Sigma) are the averages of four, five and two trials, respectively. The L-lactate concentrations reported for all other reagents are the results of single trials. Note, however, that there is replication in the data owing to the analysis of PEGs from multiple sources. For example, PEG 8000 from five sources was tested (Fluka and four screen reagents) and the five measurements yielded similar results (Table 1).

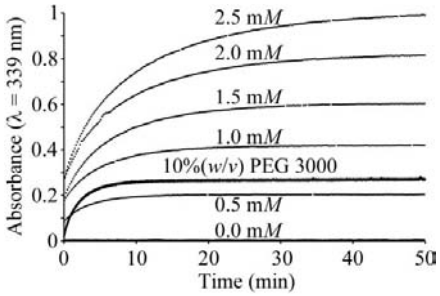

Figure 1
Enzymatic detection of L-lactate in solution. The curves show raw data from the analysis of six L-lactate standards (L-lactate concentration = 0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 m*M*) and a 10%(*w*/*v*) PEG 3000 stock solution.

3. Results and discussion

A standard calibration curve was constructed using L-lactate samples with L-lactate concentrations of 0, 0.5, 1.0, 1.5, 2.0 and 2.5 m*M*. The raw data for a typical standard curve are shown in Fig. 1. The method employed is an end-point assay in which L-lactate should be completely consumed. The end points in the present studies were typically reached within about 60 min, as shown in Fig. 1. The resulting calibration curve (not shown) demonstrated that the method in our hands exhibits good precision and linearity up to a L-lactate concentration of 2.5 m*M* and that L-lactate levels in the range 0.2–2.5 m*M* can be measured with confidence.

Several PEG reagents were analyzed, including the PEG 3000 solution used in the crystallization, heavy-atom soaking and cryoprotection studies for the *E. coli* PutA PRODH domain (Table 1). The raw data for a 10%(*w*/*v*) PEG 3000 solution (Fluka) are shown in Fig. 1. The end-point absorbance is 0.285, which corresponds to an L-lactate concentration of 0.67 m*M*. The PRODH domain was crystallized and cryoprotected in 24%(*w*/*v*) PEG 3000, so the concentration of L-lactate in the crystals used for structure determination was approximately 1.6 m*M*. This result helps explain the observation of a strong electron-density feature shaped like an L-lactate molecule near the FAD cofactor in the PRODH structure (Lee *et al.*, 2003). Given that L-lactate is a known competitive inhibitor of PRODH enzymes (Scarpulla & Soffer, 1978), the discovery of L-lactate in PEG 3000 confirms the identity and source of the active-site ligand in the PRODH structure.

Since PEG 3000 contains significant L-lactate, it is unsuitable for crystallization of PRODH without inhibitors and PRODH complexes with other inhibitors. Therefore, other PEGs were analyzed in order to find a suitable precipitating agent for future crystallization experiments (Table 1). As with PEG 3000, millimolar levels of L-lactate were measured in 50%(*w*/*v*) solutions of PEG 4000 (Fluka) and PEG 8000 (Fluka). Crystal screen reagents containing PEG 4000 and PEG 8000 also contain 1–2 m*M* L-lactate. These results are consistent with a previous measurement of 0.4 g kg^{-1} L-lactate in PEG 1000 (Pollegioni *et al.*, 2002). Interestingly, L-lactate was not detected in a 50%(*w*/*v*) solution of PEG 3350 (Sigma), nor in Index screen No. 45, which contains 25%(*w*/*v*) PEG 3350. Likewise, the two PEGMMEs analyzed (MW 2000 and 5000) contain no detectable L-lactate.

These results underscore the fact that reagents used for protein purification and crystallization may contain significant levels of contaminating species. The degree of contamination may also vary from vendor to vendor and from product to product. For example, it is interesting that PEG 3000 and PEG 4000, both from Fluka, contain significant L-lactate, yet PEG 3350 from Sigma and PEGMMEs from Fluka appear to be free of significant L-lactate contamination. Perhaps differences in the manufacturing processes of these products account for the differences in L-lactate contamination. It is also not known whether the age of the PEG solution is an important variable. While the present study certainly is not exhaustive, it does suggest that caution should be exercised when using PEGs in the study of enzymes that bind L-lactate, such as PRODH, LDH and D-amino-acid oxidase (Umhau *et al.*, 2000; Pollegioni *et al.*, 2002). Also, PEG 3350 and PEGMMEs should be useful precipitating agents in future crystallization studies of PRODH enzymes.

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