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# A thioredoxin fusion protein of VanH, a D-lactate dehydrogenase from *Enterococcus faecium*: Cloning, expression, purification, kinetic analysis, and crystallization

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## Abstract

The gene encoding the vancomycin resistance protein VanH from *Enterococcus faecium*, a D-lactate dehydrogenase, has been cloned into a thioredoxin expression system (pTRxFus) and expressed as a fusion protein. The use of several other expression systems yielded only inclusion bodies from which no functional protein could be recovered. Experiments to remove the thioredoxin moiety by enterokinase cleavage at the engineered recognition site under a variety of conditions resulted in nonspecific proteolysis and inactivation of the protein. The intact fusion protein was, therefore, used for kinetic studies and crystallization trials. It has been purified to greater than 90% homogeneity by ammonium sulfate precipitation followed by phenyl Sepharose chromatography. Based on  $k_{cat}/K_M$  for pyruvate, it is 20% as active as native VanH. Michaelis constants for NADPH, NADH, and pyruvate, of ~3.5  $\mu$ M, 19.0  $\mu$ M, and 1.5 mM, respectively, were comparable to those reported for the native VanH (Bugg TD et al., 1991, *Biochemistry* 30:10408–10415). Like native VanH, maximum activity of the fusion protein requires the presence of an anion (phosphate or acetate), however, in addition, a strongly reducing environment is needed for optimal efficacy. Competitive inhibition constants for ADP-ribose, NAD<sup>+</sup>, and oxamate have also been determined. Crystallization by hanging drop vapor diffusion produced two different crystal forms, one hexagonal and the other tetragonal. Flash-frozen crystals of the tetragonal form diffracted to 3.0  $\text{\AA}$  resolution at a synchrotron radiation source.

**Keywords:** antibiotic resistance; D-lactate dehydrogenase; *Enterococcus faecium*; enzyme kinetics; protein crystallization; vancomycin

Antibiotic resistance is an alarming specter in the treatment of bacterial infections. The widespread use of broad-spectrum anti-

biotics, coupled with the inherent adaptability and genetic promiscuity of bacteria, has compounded this problem, most prevalent in the hospital setting (Nichols & Muzik, 1992; Walsh, 1993). The matter has progressed to the extent that we are now faced with strains of highly pathogenic *Staphylococci*, which respond only to the antimicrobial agent vancomycin.

Vancomycin is a complex and unusual tricyclic glycopeptide antibiotic, MWt 1,500. Its unique and remarkable 30 year history of absence of resistant strains is related to its mode of action, in which it does not enter the cell but rather affects the integrity of the cell wall (Bugg et al., 1991b). The recent determination of the crystal structure of vancomycin (Schäfer et al., 1996) has led to further understanding of its mechanism. Vancomycin binds specifically to the terminal D-Ala-D-Ala modules of the growing peptidoglycan cell wall. By hydrogen-bonding specifically to the amide linkage of the D-Ala-D-Ala modules, a cross-linking reaction is prevented. This results in weakened cell walls that are susceptible

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**Abbreviations:** VanH.THX, VanH thioredoxin fusion protein; DTT, dithiothreitol; VRE, vancomycin-resistant *Enterococci*; LDH, lactate dehydrogenase; ADP-ribose, adenosine 5' diphosphoribose; NADH, nicotinamide adeninedinucleotide, reduced form; NAD<sup>+</sup>, nicotinamide adeninedinucleotide, oxidized form; NADPH, nicotinamide adeninedinucleotide phosphate, reduced form; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; MES, 2-N-morpholinoethanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; GST, glutathione-S-transferase.

to lysis. Full resistance to this class of antibiotic requires a complex pathway involving a number of different enzymes.

Recently, however, strains of *Enterococcus* have emerged that are unresponsive to treatment with vancomycin. These vancomycin-resistant *Enterococci* (VRE) are a double concern. Though once felt to be of little importance because of their lack of virulence, *Enterococci* have now graduated to the status of true pathogens and are responsible for approximately 5% of hospital-acquired infections in North America (Christie et al., 1994). Secondly, the ease with which bacteria transmit characteristics from strain to strain raises the threat that the much more virulent *Staphylococcus* and *Pneumococcus* microbes might acquire the same resistance and so become untreatable by any currently known drugs. Low-level resistance to vancomycin has already been exhibited by a mutant of *Staphylococcus aureus* (Milewski et al., 1996), *Staphylococcus hemolyticus*, and some strains of *Lactobacillus* and *Leuconostoc* bacteria (Schwalbe et al., 1987).

The mechanism used by VRE involves at least nine genes such as those encoded on transposable element Tn 1546 isolated from *E. faecium* BM4147 (Arthur et al., 1993; Walsh, 1993). Two of the genes code for a signaling protein (VanS), which detects the presence of vancomycin, and for a regulating protein (VanR), which controls transcription of the remaining genes in the pathway (Arthur et al., 1992; Fisher et al., 1996; Holman et al., 1993; Wright et al., 1993). Once the presence of vancomycin has been detected, the bacteria alter cell wall synthesis to avoid the vancomycin-sensitive step. Instead of a D-Ala-D-Ala dipeptide, these bacteria synthesize D-Ala-D-lactate modules to cap the growing peptidoglycan chains. Vancomycin, designed to hydrogen-bond to an amide linkage, is unable to bind to the ester group and can no longer prevent the formation of structurally sound cell walls (Bugg et al., 1991b).

The enzyme VanH is related to bacterial D-lactate dehydrogenases. Its role in the resistance pathway is to produce the D-hydroxy acid incorporated into the D-Ala-D-lactate module (Arthur et al., 1991; Bugg et al., 1991b). Following production of D-lactate, the enzyme VanA, a modified D-Ala-D-Ala ligase, forms the D-Ala-D-lactate linkage (Bugg et al., 1991a). A D-dipeptidase, VanX, specifically hydrolyzes D-Ala-D-Ala to ensure that only D-Ala-D-lactate modules are used in cell wall production (Wu et al., 1995; Wu & Walsh, 1995). Two further enzymes, VanY and VanZ, are also implicated in the resistance mechanism although not essential to it (Walsh, 1993; Arthur et al., 1994; Arthur et al., 1995). Strains of *Leuconostoc mesenteroides* and *Lactobacillus casei* have been isolated, which demonstrate an intrinsic resistance to high levels of glycopeptide antibiotics. They, too, incorporate a D-Ala-D-lactate module in peptidoglycan synthesis, but by a mechanism different from that of the *Enterococci* (Handwerger et al., 1994).

Humans use only L-lactate dehydrogenase (L-LDH) which is quite different from the D-specific enzyme. The exploitation of this distinction and of the difference in chirality of substrates could make VanH a suitable target for drug design. The solution of the *Lactobacillus pentosus* D-lactate dehydrogenase (D-LDH) structure (Stoll et al., 1996) has allowed a detailed comparison to the well-characterized L-lactate dehydrogenases (e.g., Clarke et al., 1989; Piontek & Rossmann, 1989). While the *L. pentosus* D-LDH enzyme can act as a model in discussions of VanH, one should not extend the analogy too far. There are important kinetic differences between VanH and other D-lactate dehydrogenases (Bugg et al., 1991b). First, VanH has a broader substrate specificity and is able to utilize larger substrates than other D-LDH's. Also, the VRE

enzyme prefers NADPH over NADH as a cofactor, whereas for *L. pentosus* D-LDH, the converse is true. Finally, the reaction catalyzed by VanH is essentially unidirectional, effecting the reduction of pyruvate to D-lactate. The virtual absence of a back reaction is entirely consistent with VanH's proposed synthetic role in the resistance mechanism (Bugg et al., 1991b).

As initial steps in understanding the unique characteristics of VanH at the level of atomic structure, we describe here the cloning, expression, kinetic analysis, and crystallization of VanH in the form of a thioredoxin fusion protein (VanH.THX). Crystallographic analysis of this fusion protein will also clarify whether the presence of the thioredoxin domain causes structural variations.

## Results and discussion

### Cloning and expression of VanH-thioredoxin fusion protein

Initial efforts to get high-level expression of VanH protein involved the vector pKMDL (Taguchi & Ohta, 1991), which had been used in our laboratory to express several other D-LDH's in a fully functional state and in high yields (Stoll et al., 1996; MacFarlane et al., unpubl. results). The D-LDH gene was replaced with the VanH gene and the recombinant plasmid was sequenced to verify its identity. When this approach failed, the VanH gene was subcloned into the expression vector pSE420, which produced high levels of VanH protein (approximately 13.5 mg/L) in several strains of *Escherichia coli* but segregated the protein in inclusion bodies. Multiple attempts applying a variety of resolubilization protocols to refold the protein from detergent solutions failed. It became evident that the VanH protein was being irreversibly inactivated either in the inclusion bodies or in subsequent resolubilization steps.

While several GST-fusion constructs also produced inclusion bodies, soluble, high-yield expression resulted from the Thiofusion Expression System. After purifying the pTRxFus plasmid, the VanH gene was prepared for subcloning by the addition of KpnI and PstI restriction sites. Ligation of VanH and pTRxFus gave the plasmid pTRxVH, which was used to express the soluble fusion protein VanH.THX in *E. coli* GI 724 cells in good yields.

### Purification of the VanH-thioredoxin fusion protein

VanH.THX was purified from cells containing the recombinant pTRxFus plasmid. Table 1 shows the results of the two-step purification scheme. The enzyme was produced in very good yield, giving 10 mg of pure protein per liter of cell culture. VanH.THX had a specific activity of 25 U/mg and showed a single band at 47 kDa as determined by SDS-PAGE (see Fig. 1). The apparent low purification factor as well as the large drop in total activity during the powerful chromatography step was most probably caused by the unspecific nature of the chemical reaction chosen to monitor progress. The unidirectional nature of VanH catalysis makes it impractical to probe for D-specificity, therefore *E. coli* L-LDH may have influenced results.

Attempts to cleave the thioredoxin domain from VanH using enterokinase were unsuccessful. Following the supplier's protocols, VanH.THX was incubated with enterokinase at 25°, 30°, and 37° for 1.0–24 h. All conditions resulted in nonspecific cleavage and, therefore, inactive VanH. Figure 2 shows a gel illustrating the results of limited proteolysis experiments. Since we were not able

**Table 1.** Purification of VanH.THX from *E. coli* GI724

Purification step	Protein (mg)	Activity <sup>a</sup> (units/mL)	Specific activity (units/mg)	Total units	Purification (fold)
Crude extract	220	44.6	8.11	1,784	1.0
40%( $(\text{NH}_4)_2\text{SO}_4$ ) pellet	72	64.3	16.08	1,157	2.0
Phenyl Sepharose fraction	17.6	9.9	24.75	436	3.0

<sup>a</sup>Determined by NADH-dependent reduction of pyruvate at pH 5.7 (see Materials and methods). One unit is defined as the activity required to convert 1  $\mu\text{mol}$  of substrate to product per minute.

to cleave VanH.THX and recover active, or even structurally intact enzyme, kinetic analyses and crystallization trials proceeded with the fusion protein.

#### Sequence comparison

Figure 3 shows the sequence alignment between VanH and the d-lactate dehydrogenases from *L. pentosus* and *L. mesenteroides*. Neither the *L. pentosus* or the *L. mesenteroides* enzyme contains any cysteines while VanH has six. These residues may have important implications for both VanH.THX enzymatic activity and crystallization, as indicated by the need for a reducing environment.

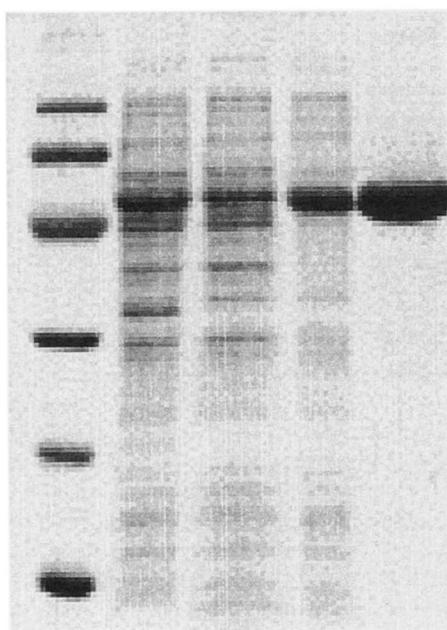
Overlaying the sequence of VanH and the known crystal structure of the *L. pentosus* d-LDH suggests that only two of the six cysteines in VanH might form disulfide bonds in the monomer. While none of the other cysteines appear to be involved in inter-

subunit disulfide bonds in the physiologically relevant dimer, it is possible that some are involved in the formation of intermolecular bonds within the crystal or interactions with the thioredoxin domain.

#### Kinetic analysis of the VanH-thioredoxin fusion protein

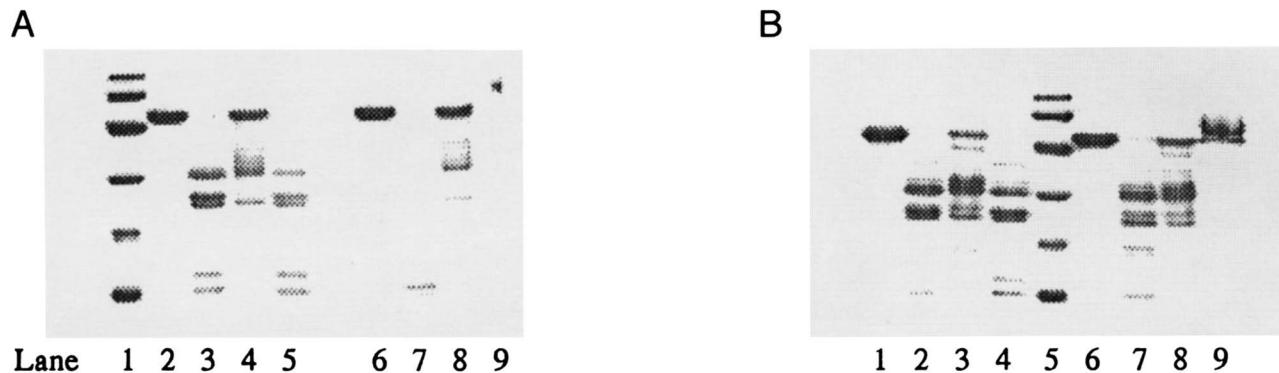
The kinetic parameters for VanH.THX were determined and compared to those of native VanH (Bugg et al., 1991b). VanH.THX maintains a preference for NADPH over NADH as the nucleotide substrate. Kinetic experiments carried out using conditions identical to those for the native enzyme in phosphate buffer at pH 5.6 showed a dramatic decrease in  $k_{cat}/K_M$ , exhibiting a 1,400-fold reduction in activity. Modification of the conditions was required to attain activity comparable to VanH alone. For example, we have determined that, like native VanH, VanH.THX activity is enhanced by the presence of an anion. Either phosphate or acetate was effective, although acetate was preferred slightly based on specific activities. The fusion protein also required a strongly reducing environment. Activity increased steadily upon addition of  $\beta$ -mercaptoethanol, up to a concentration of 200 mM. In the presence of 100 mM MES pH 5.7, 100 mM acetate and 200 mM  $\beta$ -mercaptoethanol, the  $K_M$ 's for NADPH, NADH, and pyruvate are 3.5  $\mu\text{M}$ , 19.0  $\mu\text{M}$ , and 1.5 mM, respectively. These correlate well with  $\sim 2 \mu\text{M}$ , 21  $\mu\text{M}$ , and 1.45 mM reported by Bugg et al. (1991b) for native VanH; however, the fusion protein showed only 20% of the activity of native VanH based on  $k_{cat}/K_M$  values for pyruvate. Since we expect a large domain movement in the catalytic cycle (Stoll et al., 1996), it is reasonable to suppose that the addition of thioredoxin might affect active site closure and cause slightly altered  $V_{max}$  for the fusion protein. Results of the kinetic analyses are given in Table 2.

Unlike other members of the d-LDH family, the back reaction of VanH (d-lactate to pyruvate) is extremely slow compared to the forward reaction. This is consistent with the enzyme's role in the antibiotic resistance pathway, namely to produce d-lactate for cross-linking purposes. To determine the parameters modulating the rate of reverse versus forward reaction, we determined inhibition constants for ADP-ribose,  $\text{NAD}^+$  (a product inhibitor) and oxamate (a pyruvate analog inhibitor). Inhibition constants for ADP-ribose and  $\text{NAD}^+$  with *L. mesenteroides* d-LDH were determined for comparison. Both ADP-ribose and  $\text{NAD}^+$  have  $K_i$ 's below 1 mM for the *L. mesenteroides* d-LDH at pH 7.5; VanH.THX has a comparable  $K_i$  for ADP-ribose of 0.88 mM at pH 5.7. However, VanH.THX has a  $K_i$  of 9.5 mM for  $\text{NAD}^+$  (11-fold higher than ADP-ribose with VanH.THX) at pH 5.7 and 6.6 mM at pH 7.5 (ninemfold higher than the  $K_i$  for  $\text{NAD}^+$  with *L. mesenteroides* d-LDH). This suggests that the build up of positive charge on the



**Lane**      **1**      **2**      **3**      **4**      **5**

**Fig. 1.** Purification gel. Lane 1: molecular weight markers. Lane 2: crude extract (post French press). Lane 3: supernatant. Lane 4: 40% ammonium sulfate pellet. Lane 5: purified VanH.THX.



**Fig. 2.** Proteolysis gels. Concentration of VanH.THX adjusted to 1 mg/mL in 50 mmol MES or tris, pH = 7.0, enterokinase concentration 0.3 mg/mL in H<sub>2</sub>O. Gel A: 24 h, Gel B: 12 h, lanes 1–5 at 37 °C, lanes 6–9 at room temperature. In gel A, lane 1 = molecular weight marker, lane 2 = control, lane 3 = VanH.THX + enterokinase, lane 4 = VanH.THX + urea + methylamine + enterokinase, lane 5 = VanH.THX + β-mercaptoethanol, lane 6 = control, lane 7 = VanH.THX + enterokinase, lane 8 = VanH.THX + urea + methylamine + enterokinase, lane 9 = VanH.THX + β-mercaptoethanol. In gel B, lane 1 = control (VanH.THX alone), lane 2 = VanH.THX + enterokinase, lane 3 = VanH.THX + urea + methylamine + enterokinase, lane 4 = VanH.THX + β-mercaptoethanol, lane 5 = molecular weight marker, lane 6 = control, lane 7 = VanH.THX + enterokinase, lane 8 = VanH.THX + urea + methylamine + enterokinase, lane 9 = VanH.THX + β-mercaptoethanol.

nicotinamide ring during NAD(P)H oxidation significantly decreases the enzyme's affinity for the nicotinamide dinucleotide. One way to achieve this result would be to make the electrostatic potential at the NADP<sup>+</sup> binding site more positive and thereby reduce the enzyme's affinity for it. The  $K_i$  for oxamate with VanH.THX is 2.3 mM, approximately half of that reported for the *L. pentosus* d-LDH (Taguchi & Ohta, 1994).

#### Crystallization and crystallographic analysis

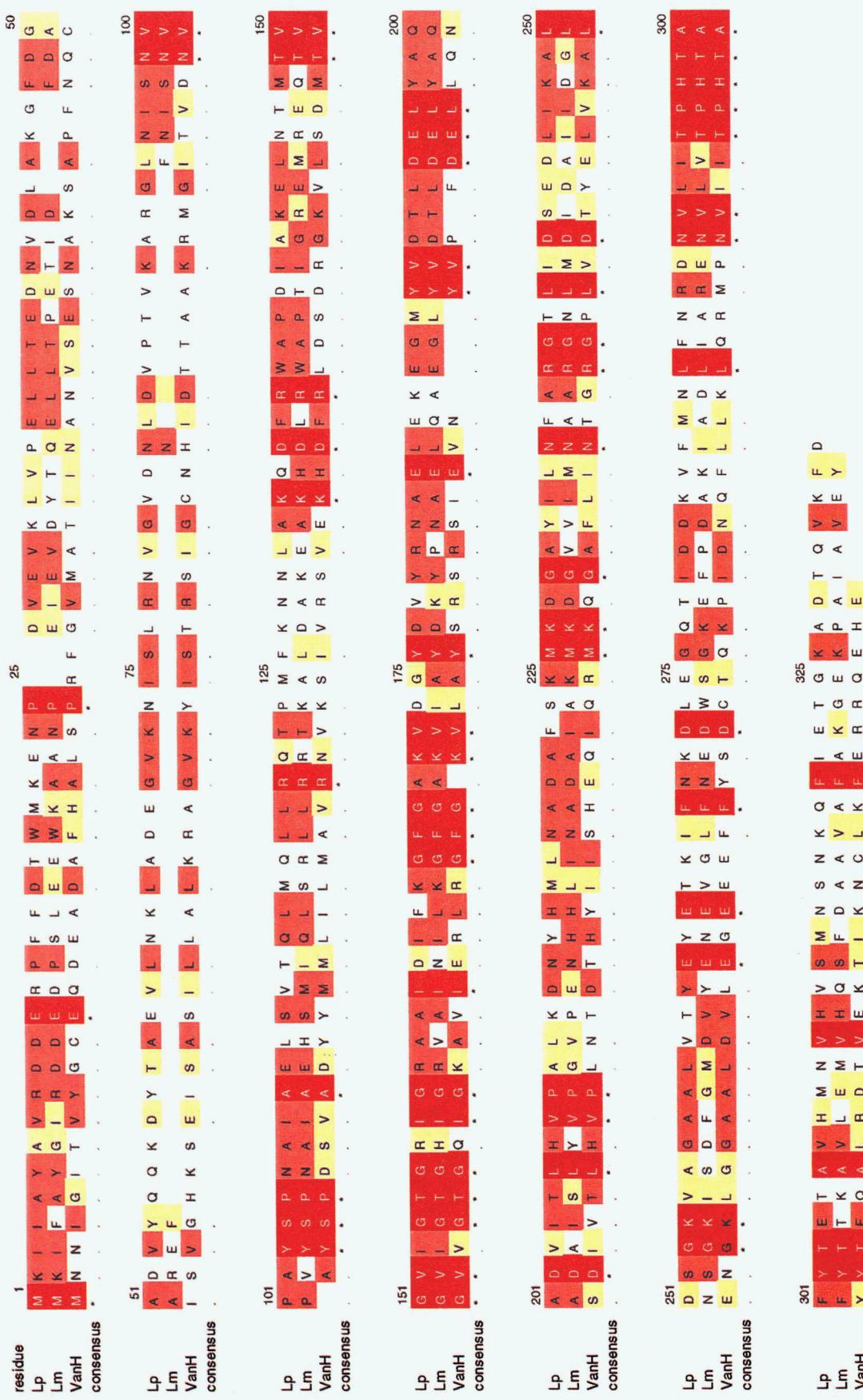
The crystals of VanH.THX grow in two different forms, one hexagonal ( $a = b = 104 \text{ \AA}$ ,  $c = 246 \text{ \AA}$ ) and the other tetragonal ( $a = b = 91.6 \text{ \AA}$  and  $c = 146.5 \text{ \AA}$ ). Both grow to a maximum size of  $600 \times 500 \times 500 \mu\text{m}$  (see Fig. 4A,C). The two forms, easily distinguishable by their external habit, grew from identical conditions and could be found in the same hanging drop. Crystal stability could be significantly improved (up to several months) if crystals were transferred to artificial mother liquor containing 200 mM β-mercaptoethanol. Unfrozen crystals diffracted to 3.8 Å resolution in a beam from a rotating anode source. Data collected before and after addition of β-mercaptoethanol showed no change in unit cell parameters and could be scaled together. Frozen crystals of VanH.THX diffracted to a maximum resolution of 2.9 Å at the A1 beamline at CHESS (Fig. 4B,D).

Crystal systems for the two forms were determined using the cell reduction algorithms of DENZO (Otwinowski, 1993a). The discrimination factor was 0.64% for the tetragonal form and 0.80% for the hexagonal form, compared to 9.85% and 3.20% for the respective next best solutions. Preliminary data collected of the hexagonal form, which diffracted less strongly, were incomplete and did not allow unambiguous assignment of the space group. Three complete data sets have been collected for the tetragonal form and the Laue group has been confirmed as P4/mmm by comparison of merging statistics. Examination of axial reflections led to the assignment of the space group as P422 (space group #89). The statistics for the three tetragonal data sets are shown in Table 3. A self-rotation function gave no noncrystallographic peaks.

Assuming  $z = 8$  gives a Matthews parameter of  $V_m = 3.27 \text{ \AA}^3/\text{kDa}$ , corresponding to a solvent content of 59% (Matthews, 1968). Crystals of related enzymes show similar or greater solvent content; 2-hydroxyisocaproate dehydrogenase crystallized with 72% solvent (Dengler et al., 1997), and glyceraldehyde-3-phosphate dehydrogenase, with 55% (Korndorfer et al., 1995). Based on the native molecular weight, and in accord with similar d-dehydrogenases (Moras et al., 1975; Goldberg et al., 1994; Lamzin et al., 1994; Stoll et al., 1996), we expect VanH (and therefore VanH.THX) to form dimers associated through the NAD-binding domains, with the two monomers related by a twofold axis. This would constrain each dimer to lie on a crystallographic twofold axis.

The solution of the VanH crystal structure would contribute to the currently sparse information about d-LDH structures. In addition, successful crystallizations of fusion proteins are still novel and we believe our study represents the first such example for a thioredoxin fusion protein. The determination of the structure of VanH.THX will provide a starting point for drug design experiments. A specific inhibitor of VanH, used in conjunction with vancomycin, could restore antibiotic sensitivity to treat infections of VRE.

In conclusion, we have cloned and expressed the vancomycin resistance protein VanH, a d-lactate dehydrogenase, as a thioredoxin fusion protein, VanH.THX. The enzyme was characterized kinetically in reactions with NADPH, NADH, and pyruvate and gave  $K_M$  values similar to those reported for VanH alone (Bugg et al., 1991b). Activity was found to be highly dependent on a reducing environment and the presence of an anion. Inhibition studies using ADP-R, NAD<sup>+</sup>, and oxamate (competing for pyruvate binding) suggest that NAD(P)<sup>+</sup> binds very poorly to the enzyme. We propose that this is the result of unfavorable interactions between the positive charge on the nicotinamide ring and the active site of VanH, which we anticipate will be more electro-positive than in other NAD-dependent d-specific dehydrogenases. We have crystallized VanH.THX in two distinct crystal forms and collected complete data sets for the tetragonal form. Efforts are currently underway to solve the crystal structure of this fusion protein.



**Table 2.** Kinetic data for VanH.THX vs. VanH, *L. mesenteroides* d-LDH and *L. pentosus* d-LDH

Substrate	VanH.THX			VanH			<i>L. mesenteroides</i> DLDH		<i>L. pentosus</i> DLDH	
	$k_{cat}$ (s <sup>-1</sup> )	$K_M$ (mM)	$k_{cat}/K_M$ (M <sup>-1</sup> s <sup>-1</sup> )	$k_{cat}$ (s <sup>-1</sup> )	$K_M$ (mM)	$k_{cat}/K_M$ (M <sup>-1</sup> s <sup>-1</sup> )	$k_{cat}$ (s <sup>-1</sup> )	$K_M$ (mM)	$k_{cat}/K_M$ (M <sup>-1</sup> s <sup>-1</sup> )	$K_i$ (mM)
Pyruvate	10.7	1.49	$7.2 \times 10^3$	40	1.45	$2.8 \times 10^4$	2680	0.075	$2.2 \times 10^7$	
NADPH		3.5			2			96		
NADH		36.6			21			8.0		
		$K_i$ (mM)						$K_i$ (mM)		$K_i$ (mM)
NAD <sup>+</sup>		$9.5 \pm 1.3$ (pH 5.7)								
		$6.6 \pm 1.0$ (pH 7.5)								
ADP-ribose		$0.7 \pm 0.2$ (pH 5.7)					$0.9 \pm 0.1$ (pH 7.5)			
Oxamate		$2.4 \pm 0.6$ (pH 5.7)							5 (pH 6.0)	

## Materials and methods

### Reagents

NADPH, NADH, NADP<sup>+</sup>, NAD<sup>+</sup>, adenosine 5'-diphosphoribose, adenosine 2'-phospho-5'-diphosphoribose, oxamate, and pyruvate were purchased from Sigma (St. Louis, MO). Enterokinase and T4 DNA ligase were obtained from Boehringer Mannheim (Laval, Quebec, Canada) and phenyl Sepharose from Pharmacia (Piscataway, NJ). The pTRxFus kit and pSE420 plasmids were obtained from Invitrogen. All other chemicals were of the highest quality commercially available.

### Cloning

Plasmid psD8 carrying the VanH gene was a gift from Dr. Christopher T. Walsh of Harvard Medical School. This plasmid expressed low levels of VanH in *E. coli* (Bugg et al., 1991b). It was recloned into a high expression vector (pKMDL) and PCR was used to generate appropriate cloning sites for the VanH gene. The pKMDL plasmid was digested with EcoRI/HindIII to release the d-lactate dehydrogenase gene and the VanH PCR product was cloned into this vector using directional cloning. Sequencing the recombinant plasmid pKVH confirmed its orientation and sequence. The VanH gene was subcloned into several expression vectors, two of which (psE420,pTRxFus) showed promising levels of expression.

To produce the thioredoxin fusion construct, the plasmid pTRxFus was digested with KpnI/PstI and the fragments separated on a 1% agarose gel. The ligated plasmid was recovered using electroelution and purified by ammonium acetate/ethanol precipitation and two 70% ethanol washes and suspended in TE buffer pH 8.0. The VanH gene was prepared for subcloning by engineering KpnI and PstI flanking restriction sites, using PCR and synthesized oligonucleotides with KpnI and PstI sites as primers for the appropriate 5' and 3' locations. The PCR product was digested with the restriction enzymes KpnI and PstI, and purified for ligation as described for pTRxFus above. The ligation was carried out using T4 DNA ligase under conditions recommended by the supplier.

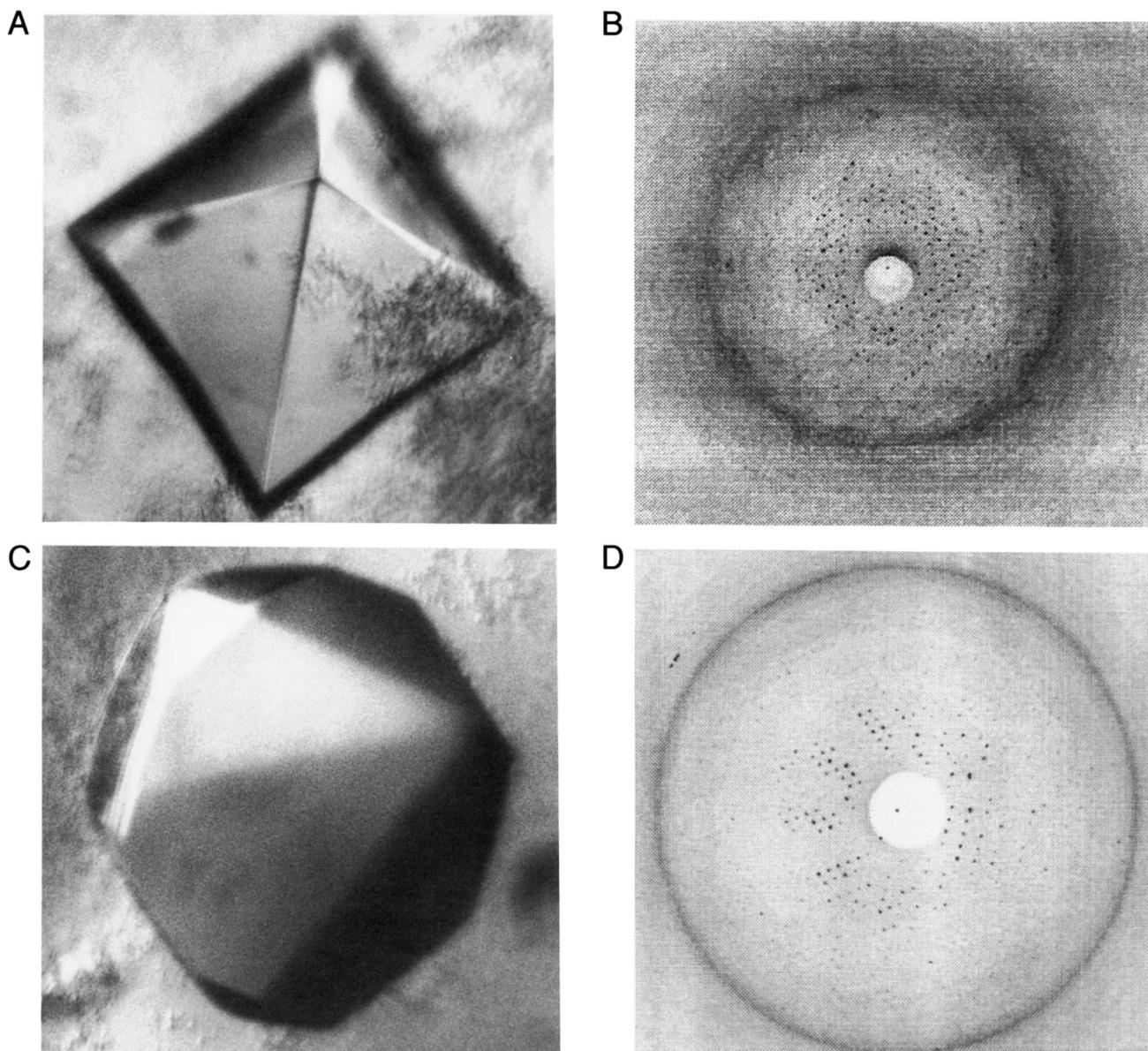
### Expression and purification

The expression of pTRxVH was carried out as described in the protocol provided by the supplier. The gene was transformed into *E. coli* GI724 cells using electroporation (400 mΩ, 25 μF, 1.8 kV). An isolated colony was used to grow an overnight culture at 30 °C in RM medium plus 100 μg/mL ampicillin. This culture was used to inoculate 1L of induction medium ( $OD_{500} \approx 0.5$ ). At log phase, expression was induced with tryptophan at a final concentration of 100 μg/mL. The culture was incubated at 37 °C and shaken at 220 rpm for an additional 4 h. Cells were harvested ( $4,500 \times g$ , 20 min, 4 °C) and resuspended in 100 mM Tris.HCl, pH 7.5, 1 mM EDTA and 5 mM DTT. All subsequent steps were carried out at 4 °C. The cells were passed twice through a French press at 12,000 psi, followed by centrifugation at  $15,000 \times g$  for 30 min. The fusion protein was precipitated from the supernatant by adding saturated ammonium sulfate solution to a final saturation of 40%, followed by centrifugation at  $15,000 \times g$  for 30 min. The pellet was resuspended in 100 mM Tris.HCl, pH 7.5, 1 mM EDTA and 5 mM DTT and loaded onto a 150 mL phenyl Sepharose HPLC column. The column was equilibrated with 20 mM Tris, pH 7.5. After a wash of two column volumes, the protein was eluted at 5 mM Tris.HCl, pH 8.8. Fractions containing VanH were concentrated to ca. 10 mg/mL and the buffer changed to 50 mM HEPES, pH 7.0, 5 mM DTT before the protein solution was stored in liquid N<sub>2</sub>.

The standard protocols for enterokinase cleavage were followed as recommended by the supplier. Reactions were carried out at 25, 30, and 37 °C. Reactions at all temperatures were followed by SDS-PAGE and VanH enzymatic activity was analysed over a time period of 1–24 h. The reactions were carried out at 1 mg/mL VanH.THX, 50 mM Tris.HCl pH 7.0 in the presence of (a) enterokinase (0.3 mg/mL) alone, (b) enterokinase plus 2 M urea and 20 mM methylamine, and (c) enterokinase plus 200 mM β-mercaptoethanol.

### Activity assays and kinetic measurements

The assay conditions of Bugg et al. (1991b) were modified to account for VanH.THX's requirement of a strongly reducing en-



**Fig. 4.** **A:** Picture of tetragonal crystal. **B:** Diffraction pattern of tetragonal form. **C:** Picture of hexagonal crystal. **D:** Diffraction pattern of hexagonal form.

vironment and the presence of divalent anions for optimal activity. Activity assays measured the decrease in absorbance of NADPH at 340 nm ( $\epsilon = 6.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ) in 100 mM MES pH 5.7, 100 mM sodium acetate, 200 mM DTT, 50  $\mu\text{M}$  NADPH and 20 mM pyruvate.  $K_M$  values were determined by varying substrate concentrations from 4 to 17  $\mu\text{M}$  NADPH, 25 to 100  $\mu\text{M}$  NADH, or 1.8 mM to 9.9 mM pyruvate. Inhibition studies were performed under the above conditions at pH 5.7 and at pH 7.5 (100 mM HEPES) at fixed variable concentrations of inhibitor. Inhibition studies of *L. mesenteroides* D-LDH were carried out at pH 7.5 using the same protocol.

Reciprocal initial velocities were plotted against the reciprocal of the variable substrate concentration and the data were fit to appropriate rate equations, assuming equal variances of  $v$ , using

the Cleland suite of kinetic programs (Cleland, 1979). Individual saturation curves were fit to Equation 1, while data for competitive inhibition were fit to Equation 2.

$$v = VA/K + A \quad (1)$$

where  $v$  = velocity,  $V$  = maximal velocity, and  $A$  = substrate concentration;

$$v = VA/K(1 + I/K_{is}) + A \quad (2)$$

where  $K$  = constant,  $I$  = inhibitor concentration, and  $K_{is}$  = inhibition constant.

**Table 3.** Data collection and reduction

Data set	I	II	III
Collected at Detector	CHESS Fuji image	CHESS Fuji image	CHESS Princeton CCD
Wavelength (Å)	0.914	0.914	0.928
No. of reflections	60,540		51,718
No. unique	10,425	11,291	8,704
Resolution limit (Å)	3.0	3.2	3.4
$R_{sym}$ (%)	7.9	13.5	5.9
% completeness	78.2	91.0	98.2
$R_{sym}$ (top bin) (%)	51	47	38
% completeness (top bin)	82.9	95.6	99.2

### Crystallization

Crystals of VanH.THX were grown by the hanging drop vapor diffusion method at room temperature. The protein solution contained 4 mg/mL in 50 mM HEPES, pH 7.0 and 5 mM DTT, and the precipitant was 0.8 M NaH<sub>2</sub>PO<sub>4</sub>, 0.4 M K<sub>2</sub>HPO<sub>4</sub>, 0.1 M HEPES. 10 μL of protein solution were mixed with 10 μL of reservoir solution and equilibrated against 1 mL of reservoir. For stabilization, fully grown crystals were transferred to artificial mother liquor (100 mM HEPES, pH 7.5 and 1.0 M NaH<sub>2</sub>PO<sub>4</sub>, 0.7 M K<sub>2</sub>HPO<sub>4</sub>, 200 mM β-mercaptoethanol).

### X-ray diffraction analysis

Crystals were mounted in thin-walled glass capillaries (Supper Co, Natick, MA). Preliminary data sets were collected on a Siemens X1000 detector using Cu-Kα radiation from a rotating anode (RU200H, Rigaku; 0.2 × 2 mm<sup>2</sup> focus; 37 kV, 70 mA), Ni-filtered and focused with Francks mirror optics. Data were reduced using an updated version of the program package XDS (Kabsch, 1988). Higher resolution data for the tetragonal form were collected at the synchrotron radiation source at Cornell University (CHESS) using Fuji image plates. The wavelength used was 0.91 Å. Crystals for these experiments were frozen in the nitrogen stream at -173°C, in artificial mother liquor containing 200 mM β-mercaptoethanol and 25% glycerol. Preliminary data for the hexagonal crystal form were also collected at CHESS, again using frozen crystals but with an ADSC 2K CCD detector system. All synchrotron data were processed, scaled, and cell dimensions refined using the HKL package (Minor, 1993; Otwinowski, 1993a, 1993b).

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