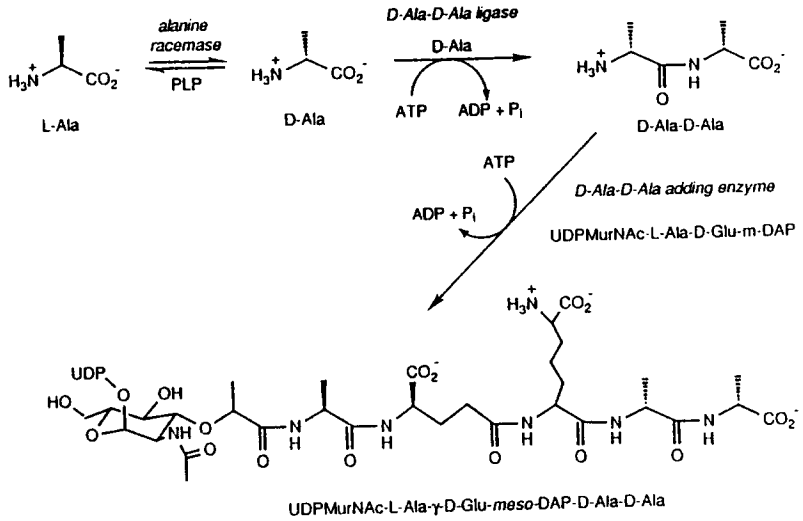
Molecular Studies on the Genes and Enzymes of the D-Alanine Branch of Bacterial Peptidoglycan Biogenesis



Peptidoglycan Biogenesis as a Target for Antibiotics

Enzyme	Antibiotic
Cytoplasmic Phase	
Alanine racemase	β-F-D-Alanine, Ala-P
D-Ala-D-Ala ligase	D-Cycloserine (+ Phosphinate analog of D-Ala-D-Ala)
D-Ala-D-Ala adding enzyme	None
Membrane Phase	
Undecaprenyl-PP transferase	Bacitracin
Cross-linking transpeptidase	β-Lactams (penicillin, cephalosporin)
Binding of peptidyl-D-Ala-D-Ala	Vancomycin, ristocetin

To study enzyme mechanism: first need pure enzyme in substantial (mg) quantity.

Strategy:

1. Clone gene.
2. Sequence gene.
3. Construct an overproducing vector.
4. Purify to homogeneity.
5. Characterize for mechanistic/structural studies.

Two Approaches to Cloning:

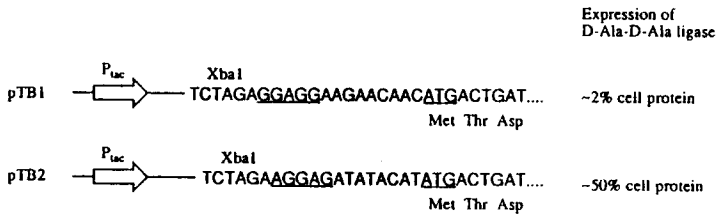
1. Hybridisation
   - purify a small amount of protein
   - obtain N-terminal sequence
   - make ^{32}P-labelled complementary oligonucleotide probe
   - hybridise probe to gene; clone.

2. Complementation
   - obtain a mutant strain conditionally defective for protein product
   - under non-permissive conditions complement phenotype with DNA library
   - select for complementing DNA; clone.

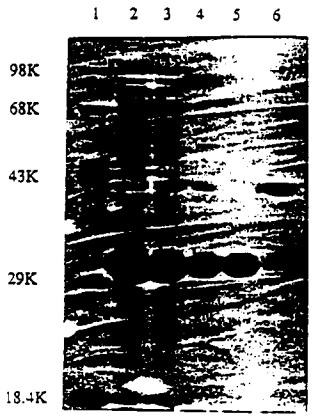
D-Ala-D-Ala ligase:
- *E. coli* ST640, temperature-sensitive *ddl*$^{-}$ strain (chemical mutagenesis)
- Complementation of ST640 with *E. coli* λ library gives two cloned genes

⇒ Two D-Ala-D-Ala ligases in E. coli?

Re-Engineering of Ribosome Binding Site



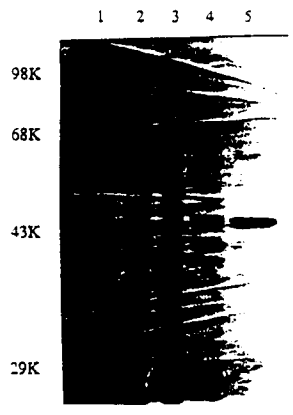
Purification of *E. coli* D-Ala-D-Ala Ligase from W3110/pTB2



Lane 1: Molecular weight markers
Lane 2: Crude Extract
Lane 3: Ammonium sulphate (25-50%) ppt.
Lane 4: AcA54 Gel Filtration
Lane 5: MonoQ FPLC
Lane 6: *S. typhimurium* D-Ala-D-Ala Ligase

	Vol (ml)	Protein (mg/ml)	Total (mg)	units (u/ml)	Sp.Activity (u/mg)	Yield (%)	Purification (fold)
Crude Extract	20.9	42.6	890	579	13.6	100	1.0
$(NH_4)_2SO_4$ ppt.	11.0	64.0	704	1084	16.9	98.5	1.24
Gel Filtration	64.0	6.7	430	205	30.6	108.5	2.25
MonoQ FPLC	48.0	6.8	326	210	31.0	83.4	2.28

Purification of *E. coli* D-Ala-D-Ala Adding Enzyme from JM105/pTB4



Lane 1: Molecular weight markers
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Strategy for Enzyme Mechanistic Study

Determination of Kinetic Mechanism - Order of addition of substrates, release of products.

Determination of Chemical Mechanism - Order of bond-breaking and bond-making steps, search for intermediates

Both aspects of enzyme mechanism can affect inhibitor design.

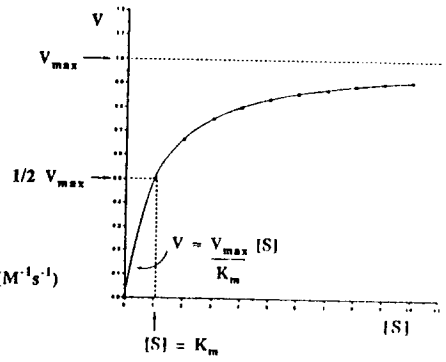
$$E + S \rightleftharpoons E.S \xrightarrow{k_{cat}} E.P \rightleftharpoons E + P$$

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

$V_{max} = k_{cat}[E_{total}]$

k_{cat} = turnover number (sec^{-1})

$\frac{k_{cat}}{K_m}$ = enzymatic catalytic efficiency ($M^{-1}s^{-1}$)

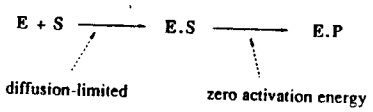


⇒ can experimentally measure V_{max}, K_m

Upper Limit for Catalytic Efficiency

Every E + S encounter gives turnover to E + P

⇒ catalysis limited by rate at which E + S diffuse together



Diffusional upper limit ≈ 10^8-10^9 $M^{-1}s^{-1}$

$$\Rightarrow \frac{k_{cat}}{K_m} \leq 10^8\text{-}10^9\ M^{-1}s^{-1}$$

Catalytic Efficiencies of Some Highly Efficient Enzymes

Enzyme	Substrate	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m ($M^{-1}s^{-1}$)
Acetylcholinesterase	Acetylcholine	1.4×10^4	9×10^{-5}	1.6×10^8
Triose phosphate isomerase	Glyceraldehyde-3-P	4.3×10^3	4.7×10^{-4}	2.4×10^8
β-Lactamase	Benzylpenicillin	2.0×10^3	2×10^{-5}	1×10^8
and some less efficient ones....				
Alanine racemase	L-Alanine	14.3	2×10^{-3}	7.2×10^3
D-Ala-D-Ala ligase	D-Alanine	10.7	6×10^{-4}	1.8×10^4
D-Ala-D-Ala adding enzyme	D-Ala-D-Ala	13.1	2.2×10^{-4}	6.0×10^4

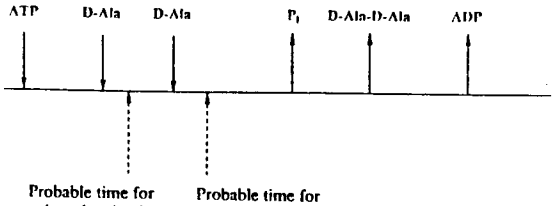
Kinetic Mechanism of D-Ala-D-Ala Ligase

$$\text{D-Ala + D-Ala + Mg.ATP} \xrightarrow[ligase]{\textit{D-Ala-D-Ala}} \text{D-Ala-D-Ala + Mg.ADP + } P_i$$

- three substrates/three products.
- ordered vs. random binding and release
- are some products formed and released before all substrates bind?

Ordered ter-ter mechanism:

(deduced from steady state kinetics, substrate/product inhibition)



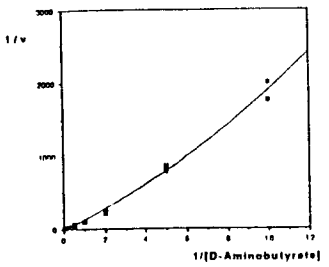
Kinetic Analysis of Two D-Alanine Binding Sites

- D-alanine used as both the electrophilic and nucleophilic partners in peptide bond formation.

- Two identical molecules of D-alanine bound in distinct binding sites with very different affinities.

$$E + S \underset{}{\overset{K_1}{\rightleftharpoons}} ES + S \overset{K_2}{\rightleftharpoons} ES_2 \xrightarrow{k_{cat}} E + P$$

$$v = \frac{V_{max}[S]^2}{K_1K_2 + K_2[S] + [S]^2}$$

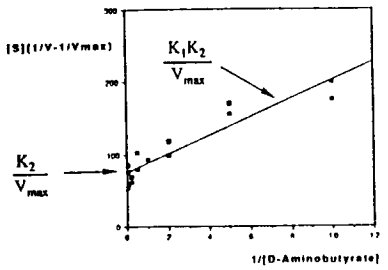


K_1 = 2-6 μM

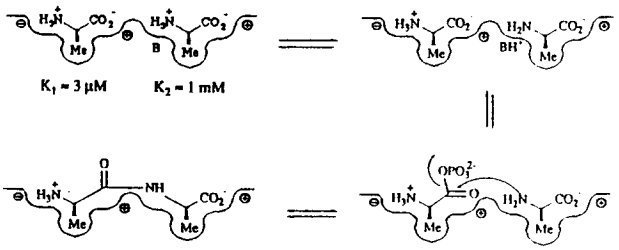
K_2 = 540-1200 μM (= apparent K_m)

⇒ First D-Ala bound 250-500x more tightly than second D-Ala

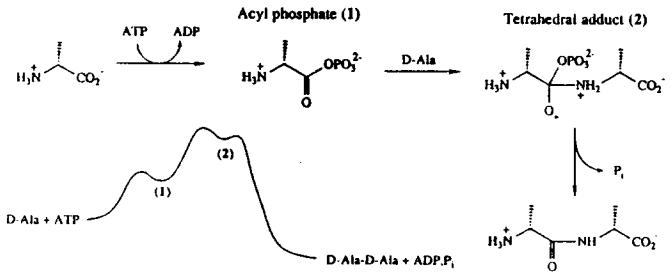
⇒ Specificity leakage at C-terminal D-alanine site? (product D-Ala-D-Xaa)



Proposed Two Subsites for D-Alanine Recognition



Mechanism of D-Ala-D-Ala Ligase



To study reversible formation of (1):

A. Positional Isotope Exchange (PIX)
B. Molecular Isotope Exchange (MIX)

To study reversible formation of (2):

- make stable analogs of transition state or reaction intermediate; test as inhibitiors.
- CPMAS solid state NMR of enzyme-inhibitor complex.

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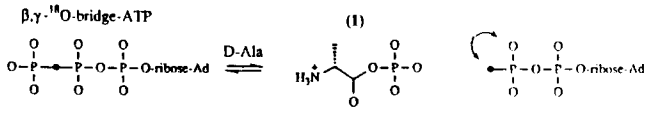

Assay by ^{18}O shift on ^{31}P NMR signal of γ-phosphate of ATP.

^{18}O scrambles faster than forward reaction rate:

Kinetic competence vs. chemical competence.

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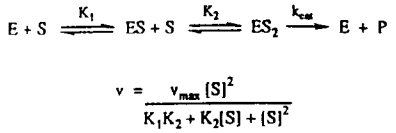

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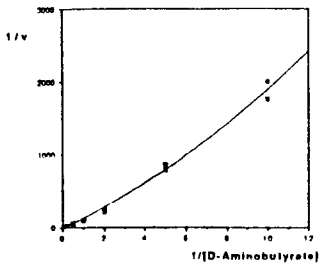

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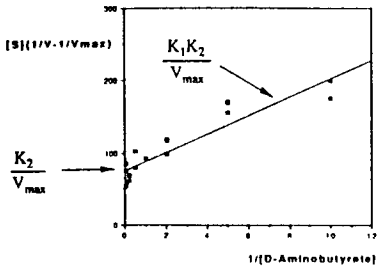

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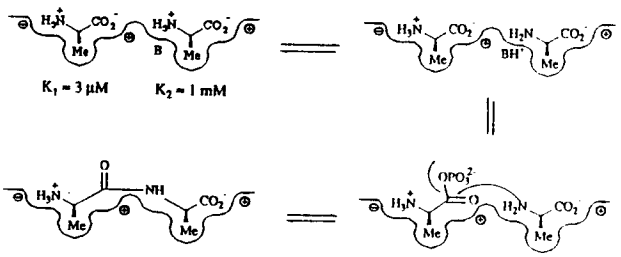
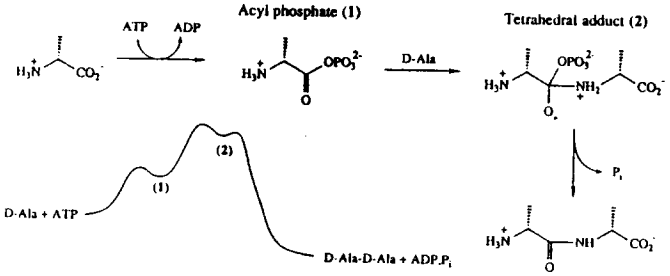


Mechanism of D-Ala-D-Ala Ligase



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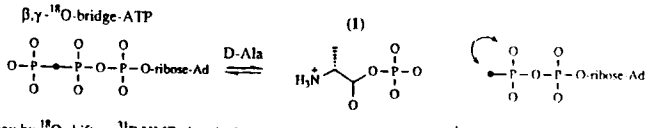

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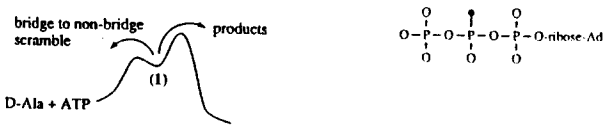


Kinetic competence vs. chemical competence.

Molecular Isotope Exchange (MIX)

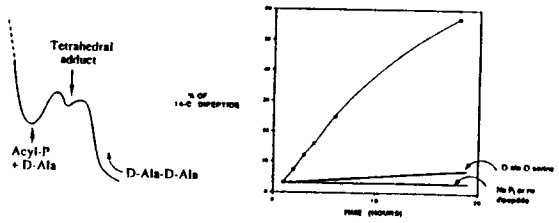
Partial back reaction in absence of ADP (phosphorolysis of the amide bond):

D-Ala-D-Ala + P_i ⇌ D-alanyl-P + D-Ala ⇏ ATP

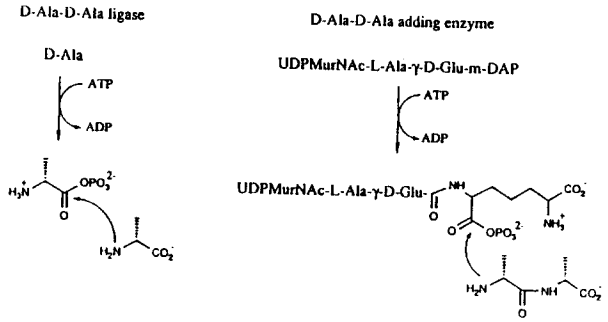
↓ release into solution

Add a trace of ^{14}C-D-Ala

⟹ Enzyme catalyzes exchange of ^{14}C-D-Ala into D-Ala-D-Ala

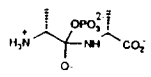


Acyl Phosphoric Anhydrides as Intermediates in ATP-Cleaving Amide Bond Formation

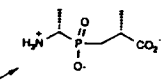


Investigation of Enzymatic Transition State

Proposed transition state



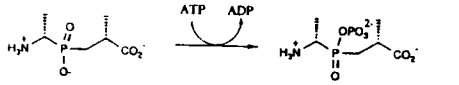
Synthesize stable analogs:



(Patchett et al., Merck)

Potent inhibitor that becomes time-dependent in assays containg ATP

Proposed mechanism:

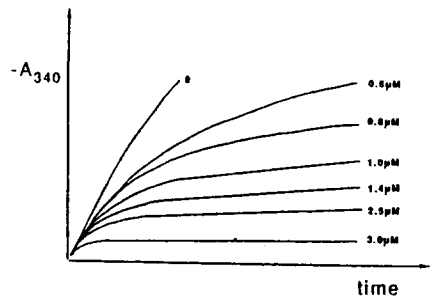


analog to tetrahedral adduct

phosphinate

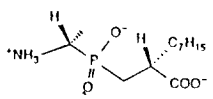
better analog to tetrahedral adduct

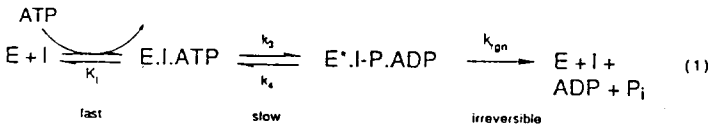
phosphinophosphate



D-ALA-D-ALA LIGASE ACTIVITY

Progress curves for time-dependent onset of reduced steady state activity in the presence of:



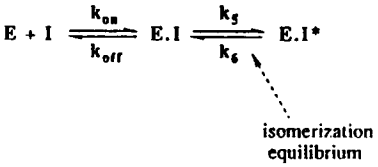


K_d vs. Binding Energy

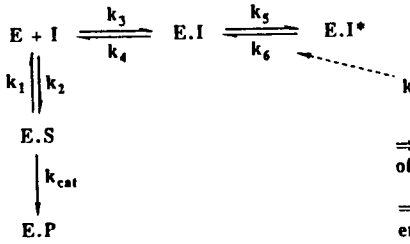
$\Delta G^\circ = -RT\ln K_{eq}$

for $K = 10^{-5}$ M ΔG = -7 kcal/mol
for $K = 10^{-8}$ M ΔG = -11.2 kcal/mol
for $K = 10^{-11}$ M ΔG = -15.4 kcal/mol

10^{-4}-10^{-5} M = normal range for substrate K_m
10^{-7}-10^{-9} M = range for specific, potent enzyme inhibitors
10^{-10}-10^{-12} M = range for "slow binding" inhibitors

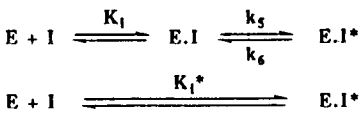


Slow-Binding Inhibition



Kinetic Constants of some Slow-Binding Inhibitors

Enzyme	Inhibitor	K_i (M)	K_i^* (M)	k_5/k_6	$t_{1/2}$ (regain)
Acetolactate synthase	sulfometuron methyl	6.6×10^{-7}	6.5×10^{-8}	10	30 min
Dihydrofolate reductase	methotrexate	3.6×10^{-9}	1.4×10^{-11}	270	27 min
Pepsin	pepstatin	1.3×10^{-8}	4.5×10^{-11}	290	2.5 hr
Alanine racemase	Ala-P	1×10^{-3}	1×10^{-9}	10^6	20 days
D-Ala-D-Ala ligase	aminoalkyl phosphinate	1.4×10^{-4}	ND	ND	17 days



How to detect a labile, enzyme-bound phosphinophosphate?

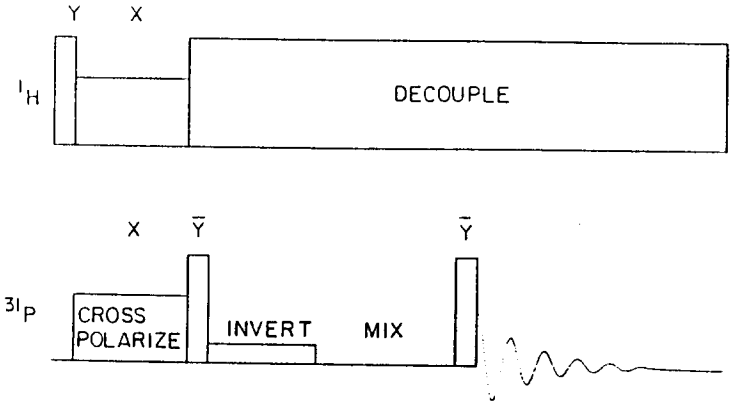
——→ NMR of enzyme-bound species

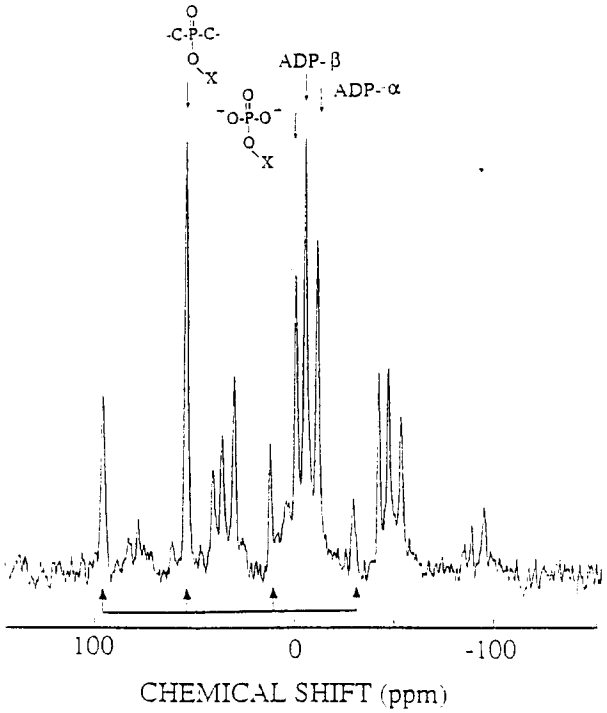
solution NMR ——→ signals too broad to be useful

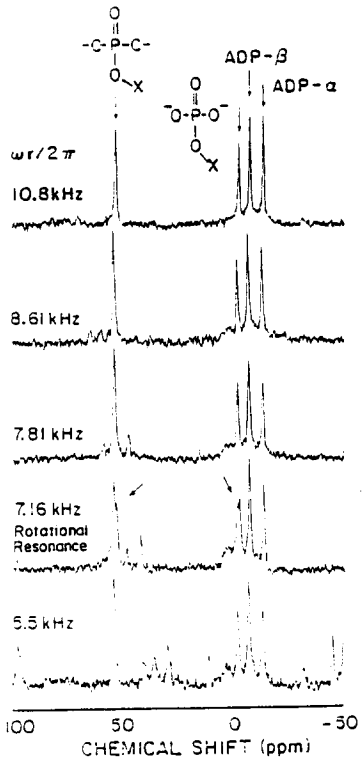
solid state NMR ——→ CPMAS ^{31}P NMR to get narrow lines

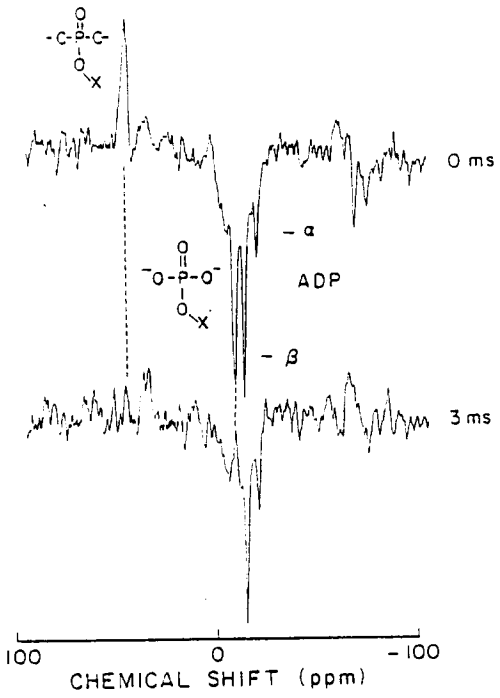
(Cross Polarization Magic Angle Spinning NMR)

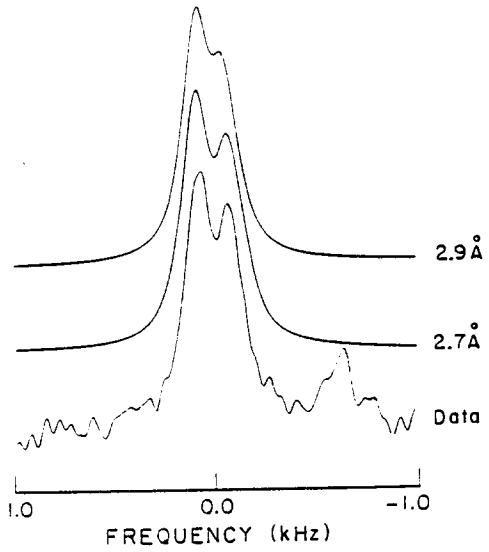
(with A. McDermott, R. Griffin, National Magnet Lab, M.I.T.)


Y
X
^1H
DECOUPLE
X
$\bar{Y}$
$\bar{Y}$
^{31}P
CROSS POLARIZE
INVERT
MIX

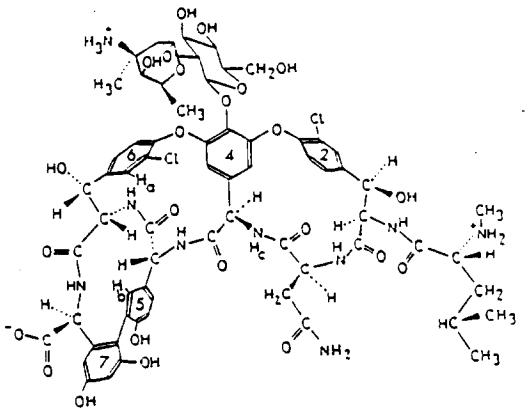

ADP-β
ADP-α
100
0
-100
CHEMICAL SHIFT (ppm)


ADP-β
ADP-α
ωr/2π
10.8kHz
8.61kHz
7.81kHz
7.16kHz
Rotational Resonance
5.5kHz
100
50
0
-50
CHEMICAL SHIFT (ppm)

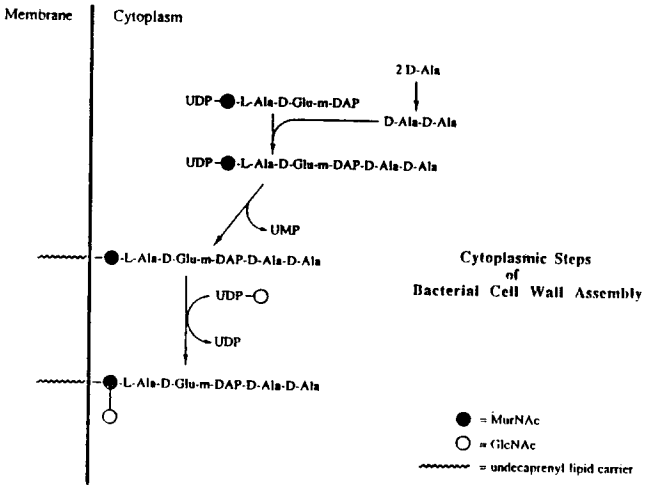

0 ms
- α
ADP
- β
3 ms
100
0
-100
CHEMICAL SHIFT (ppm)



Vancomycin Group of Glycopeptide Antibiotics

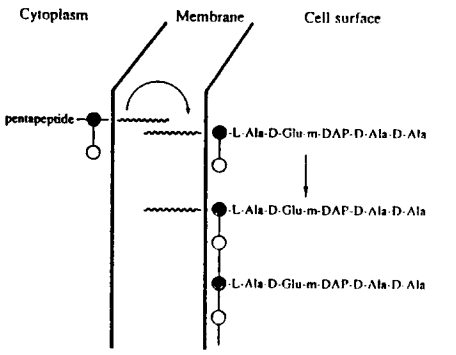


Examples: Vancomycin, ristocetin, teicoplanin
Target: Surface peptidyl chains terminating in D-Ala-D-Ala
Mechanism: Tight complexation of vancomycin to peptidyl-D-Ala-D-Ala termini, preventing both translocation and cross-linking
Clinical use: Life-threatening infections in hospital settings (e.g. multiple drug-resistant *Streptococci*, *Enterococci*)

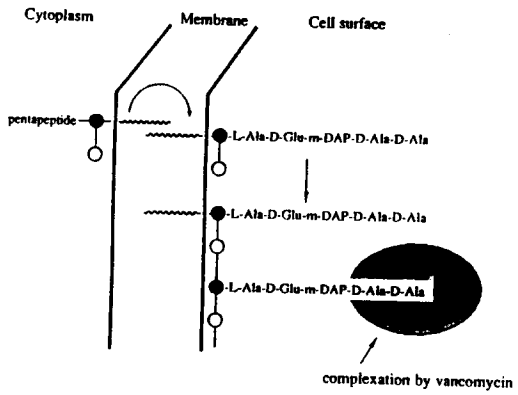


Cytoplasmic Steps of Bacterial Cell Wall Assembly

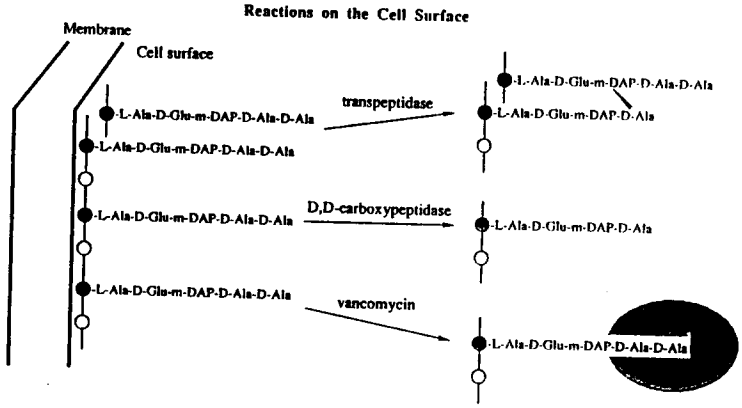
Translocation of the Cell Wall Precursor Across Cell Membrane



Translocation of the Cell Wall Precursor Across Cell Membrane



Reactions on the Cell Surface



Emerging Resistance to Vancomycin

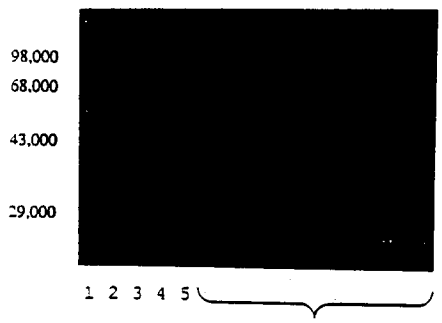
1986 Glycopeptide-resistant strains of *Enterococci* first isolated in France & U.K.

1988 Vancomycin resistance found to be plasmid-encoded. Origin of plasmids unknown.

1990 *VanA* gene subcloned from resistance plasmid, found to correlate with vancomycin resistance.
VanA gene sequenced, revealed substantial homology to *ddl* genes.

Is VanA a D-Ala-D-Ala ligase?

Purification of VanA from JM105/pAT214
(with M. Arthur, P. Courvalin, Pasteur Inst., Paris)



Fractions eluted from AcA54
gel filtration column with 1M NaCl

Lane 1: MW standards
Lane 2: Crude extract
Lane 3: Membrane fraction
Lane 4: 25%-50% Ammonium sulphate fraction
Lane 5: 50% Ammonium sulphate supernatant

% Amino Acid Sequence Homology Between VanA and D-Ala-D-Ala Ligases

	VanA	*S.typh.* DdlA	*E. coli* DdlB
Predicted MW	37,400	39,271	32,840
VanA	-	36%	28%
S. typh. DdlA	36%	-	33%
E. coli DdlB	28%	33%	-

Kinetic Parameters of VanA

		E. coli DdlA	*E. coli* DdlB	VanA
D-alanine	k_{cat}	444 min^{-1}	1018 min^{-1}	228 min^{-1}
	K_1	5.7 μM	3.3 μM	3.4 mM
	K_2	0.55 mM	1.2 mM	38 mM
	k_{cat}/K_m	1.3 x 10^4 $M^{-1}s^{-1}$	1.4 x 10^4 $M^{-1}s^{-1}$	1.0 x 10^2 $M^{-1}s^{-1}$
D-cycloserine	K_i	8.9 μM (C)	27 μM (C)	730 μM (C)
Aminoalkylphosphinate	K_i	130 μM (C)	28 μM (C)	430 μM (C)
	k_{inact}	2.4 min^{-1}	2.3 min^{-1}	4.9 min^{-1}

Comparison of VanA and D-Ala-D-Ala Ligases

Similarities

Similar size (37,000 vs. 32,000/39,000)

28-36% amino acid sequence homology

VanA possesses D-Ala-D-Ala ligase activity

VanA susceptible to inhibition by D-cycloserine and transition state analog for D-Ala-D-Ala ligase (of clinical use?)

Differences

VanA much more hydrophobic protein (stabilised by high salt conditions)

VanA probably membrane-associated, whereas D-Ala-D-Ala ligases are cytoplasmic

VanA has 1% catalytic efficiency of DdlA/B

K_m, K_I values for VanA 10-1000 fold higher than for D-Ala-D-Ala ligases

Dilemma

How might VanA as a "low efficiency", membrane associated D-Ala-D-Ala ligase impart vancomycin resistance?

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D-Ala-D-Ala Ligase

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D-Ala-D-Ala Adding Enzyme

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VanA

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