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Engineering yeast alcohol dehydrogenase. Replacing Trp54 by Leu broadens substrate specificity

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Analysis of a crystal structure of alcohol dehydrogenase (Adh) from horse liver suggests that Trp54 in the homologous yeast alcohol dehydrogenase prevents the yeast enzyme from efficiently catalysing the oxidation of long-chain primary alcohols with branching at the 4 position (e.g. 4-methyl-1-pentanol, cinnamyl alcohol). This residue has been altered to Leu by site-directed mutagenesis. The alteration yields an enzyme that serves as an effective catalyst for both longer straight-chain primary alcohols and branched chain alcohols.

Key words: alcohol dehydrogenase/crystal structure/site-directed mutagenesis

Introduction

The homologous alcohol dehydrogenases (Adhs) from horse liver and yeast differ significantly both in their rates of turnover and substrate specificities. The specific activity of the Adh from horse liver is two orders of magnitude lower with ethanol than the Adh from yeast (Hadorn *et al.*, 1975). The horse liver enzyme, however, accepts a wide range of substrates, including secondary alcohols, cycloalkanols and primary alcohols with branching at the 2, 3 and 4 positions (Jones and Beck, 1976). In contrast, Adh from yeast accepts ethanol, some short straight-chain primary alcohols and little else. Secondary alcohols are either accepted poorly (as with 2-propanol) or not at all. Primary alcohols with branching at the 2, 3 and 4 positions are also poor substrates.

When using alcohol dehydrogenases as synthetic tools, the combinations of properties in these two enzymes is unfortunate. The narrow substrate specificity of the enzyme from yeast makes it applicable as a synthetic tool for only a very small number of synthetic problems, mostly involving the preparation and analysis of isotopically substituted versions of the natural substrates (Günther *et al.*, 1973). In contrast, the Adh from horse liver is used for many synthetic applications (Prelog, 1964; Jones and Beck, 1976), where its broad substrate specificity enables it to handle a wide variety of substrates, albeit with rather low rates.

We have recently studied how variation in individual amino acids in Adh controls various aspects of catalytic specificity (Ellington, 1988; Fitz, 1988; Weinhold, 1991; Weinhold *et al.*, 1991). This has complemented work in other laboratories where site-directed mutations have been introduced into genes for either yeast or mammalian Adh proteins (Murali and Creaser, 1986; Ganzhorn *et al.*, 1987; Ganzhorn and Plapp, 1988; Creaser *et al.*, 1990; Gould and Plapp, 1990; Fan *et al.*, 1991; Höög *et al.*, 1992; Park and Plapp, 1992; Bhanson *et al.*, 1993; Green *et al.*, 1993). Underlying mutagenesis work in yeast Adh is a structural model for the protein extrapolated from the crystal structure of the enzyme from horse liver (Eklund *et al.*, 1981, 1982) based on an alignment of the protein sequences (Jörnvall *et al.*, 1987; Sun and Plapp, 1992). From such comparisons, Brändén *et al.* (1975) suggested that smaller side chains at positions 48 (Ser in horse liver Adh, corresponding to Thr45 in yeast Adh) and 93 (Phe in horse liver Adh, corresponding to Trp92 in yeast Adh) might be responsible for a larger active site in the horse liver enzyme, which in turn might explain why the liver enzyme accepts larger substrates.

Showing the general validity of this suggestion, Creaser *et al.* (1990) replaced Thr45 in yeast Adh by amino acids with smaller side chains. They found that the Thr45Ser mutant was a better catalyst for the oxidation of several larger substrates, in particular (*S*)-2-octanol, without having diminished catalytic power towards ethanol. Green *et al.* (1993) replaced Trp92 by Ala and found that the mutant was a much better catalyst for longer primary alcohols as well as primary alcohols branched at the 2 and 3 positions.

As part of a programme to develop analogues of nucleic acids for antisense work (Huang *et al.*, 1991), we wanted to oxidize some long-chain primary alcohols with branching at the 4 position which are poor substrates for yeast Adh. The analysis of Brändén *et al.* (1975) suggested that modifying one of four amino acid side chains (Trp54, Leu116, Met270 and Ile290, yeast numbering) at the entrance to the active site might improve activity towards such substrates. At positions 116 and 290, the residues in yeast Adh are the same in horse liver Adh. Met270 is replaced by Val in the enzyme from horse liver. Furthermore, in the region of Trp54, a deletion has occurred in a region that forms an active site coil between a helix and a β-strand. Depending on the alignment, W54 is either replaced by the smaller Leu side chain in horse liver Adh or deleted entirely.

Position 54 (Trp in yeast, corresponding to Leu57 in horse liver Adh) appeared to be the more interesting of the two. First, the structural model (Figure 1) suggested that the side chain of Trp54 in yeast Adh might lie at precisely the position needed to account for the low catalytic activity of yeast Adh with primary alcohols branched at the 4 position. Next, the size of the side chain (yeast Adh versus horse liver Adh) is changed more dramatically at position 54 than at position 270. Furthermore, contemporary mutation matrices (Gonnet *et al.*, 1992) suggest that substitution of Trp by Leu (found at position 54 in yeast and position 57 in horse) is less probable than the Met for Val substitution found at position 270. Finally, experiments by Wills *et al.* (1982), selecting for mutants of yeast Adh less able to accept allyl alcohol, identified variability at position 54 (substitution by Arg) as one of a few positions that had this effect.

Therefore, we have examined the effect of substituting Trp54 by Leu in constraining the substrate specificity of yeast Adh.

Materials and methods

Enzymes and materials for M13 mutagenesis and sequencing of DNA were from New England Biolabs, Pharmacia and

United States Biochemical. Small molecular weight substrates were from Fluka and were distilled prior to use. The gene encoding Adh 1 from *Saccharomyces cerevisiae* was a generous gift from E.T.Young and B.D.Hall (Williamson *et al.*, 1980).

Construction of the W54L mutant

The gene encoding Adh 1 was extracted from its carrier plasmid as a *Sph*I fragment and inserted into the polylinker sequence of the bacteriophage M13mp19 using standard procedures (Sambrook *et al.*, 1989). The orientation of the inserted Adh 1 gene was determined by restriction digestion (*Hin*dIII). The mutant gene was constructed by oligonucleotide-directed mutagenesis in *Escherichia coli* using a selection based on the asymmetrical methylation of GATC sequences in a heteroduplex (Marmenout *et al.*, 1984). Oligonucleotides were synthesized on an Applied Biosystems Automated DNA synthesizer using phosphoramidite chemistry and purified by standard methods. The mutagenesis experiments are described in detail elsewhere (Weinhold, 1991). The identity of the wild type and mutant Adh was verified by total DNA sequencing.

Protein expression

The wild type and mutant genes were subcloned into the pSL expression vector described in detail elsewhere (Ellington, 1988). The pSL vector was constructed from a fragment of the 2 μ circle and bears both a yeast Trp 1 gene as a selection marker in yeast and a gene conferring ampicillin resistance as a selection marker in *E.coli*. The wild type and mutant genes were inserted into the pSL vector using a restriction fragment obtained by *Sph*I digestion of the double-stranded form of the M13 vector. The *Sph*I fragment carries the Adh I promoter sequence, which directs the expression of the Adh genes in the constructs reported here. Successful insertions were identified by gel electrophoresis of plasmids picked from single colonies.

A mutant strain, designated RS1-1, of *S.cerevisiae* deficient in genes for both Adh 1 and Adh 2, described elsewhere (Elllington, 1988), was used as an expression host. No Adh activity was detectable in extracts from this strain, which grew extremely slowly. Introduction of the Adh 1 or mutant gene on a vector using the lithium acetate method (Ito *et al.*, 1983) yielded strains that grew much faster, presumably due to the catalytic activity of the Adh proteins encoded by the vector. The yeast strains were grown at 30°C on minimal medium [4% glucose, 0.5% $(NH_4)_2SO_4$, 0.17% yeast nitrogen base without amino acids, Difco, 0.05% $MgSO_4$, 0.03% $CaCl_2$, with 8.3 ml/l of a separately autoclaved solution containing 0.36% lysine, 0.24% histidine and 0.24% arginine] to an optical density (600 nm) of unity and the cells recovered by centrifugation (1200 *g*) and stored frozen.

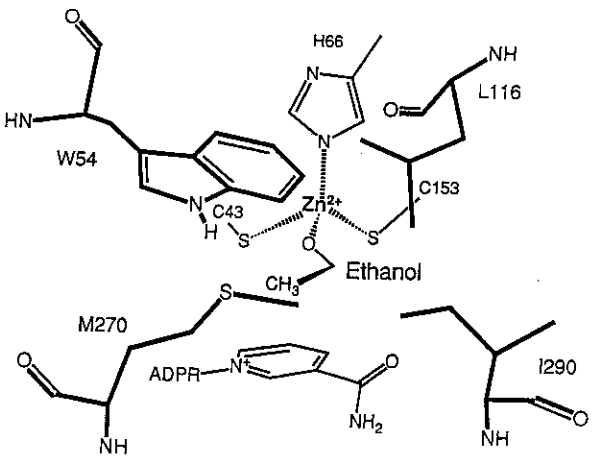

Fig. 1. Structural model for the active site of alcohol dehydrogenase from yeast built by extrapolation from the crystal structure for the homologous enzyme from horse liver. Numbering is from the yeast alcohol dehydrogenase.

Total DNA was then isolated (Rothstein, 1985) from a small portion of the yeast (50 mg) and used to transform *E.coli*. Transformants were grown, the plasmids isolated and the identity of the Adh genes verified again by total DNA sequencing.

Protein purification

Protein concentrations were determined using the Bradford (1976) test. To calibrate the Bradford test, protein absorbence at 280 nm of wild type Adh purified to homogeneity was measured and compared with the published extinction coefficient for yeast Adh (Hayes and Velick, 1954). Catalytic activities were measured by standard kinetic methods (see below). To isolate purified wild type and mutant protein, yeast (~2 g) was suspended at 4°C in potassium phosphate buffer (5 ml, 100 mM, pH 7.0, containing 1 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride and 1% propanol) and homogenized in a bead beater (BioSpec Products). The beads were recovered by filtration and the filtrate centrifuged (8000 *g*). The supernatant was treated with protamine sulfate (to 0.15%), incubated at 0°C (15 min) and the mixture centrifuged (8000 *g*). An 'AffiGel Blue' affinity column (Bio-Rad, Cibacron Blue F3GA on agarose, binding capacity 24.5 mg albumin/ml, 35 ml total volume) was then prepared by prewashing with a solution of urea (deionized, 6 M), NaCl (2 M) and pre-equilibrating with potassium phosphate buffer (10 mM, pH 7.0 containing 1 mM dithiothreitol). The supernatant of the protamine sulfate precipitation was then applied at 4°C to this column, the column washed with pre-equilibrating buffer (200 ml) and the Adh activities eluted with a gradient of NADH (0–0.2 mM) in pre-equilibrating buffer. Both wild type and mutant protein eluted at approximately [NADH] = 0.045 mM.

The fractions containing active proteins were concentrated to 2 ml by ultrafiltration (Amicon PM-10 membrane) and then to 0.2 ml via centrifugation with a Centricon 10 filter at 4°C. The concentrated protein solutions were then applied to an HPLC gel filtration column (LKB GlasPac TSK-G3000SW, 8×300 mm) and eluted with a potassium phosphate buffer (10 mM, pH 7.0 containing 300 mM Na_2SO_2 and 1 mM dithiothreitol). Fractions containing enzyme were identified by their absorbance at 280 nm. Samples were stored at 4°C. Approximately 1 mg of pure wild type and 0.5 mg of mutant protein were obtained from 2 g of cells using this procedure (Weinhold, 1991). The absorbance ratio (280/260 nm) of the mutant protein (1.39) was diminished slightly from that observed in the wild type protein (1.43) reflecting the substitution of a Trp by a Leu.

Co-factor purification and steady state kinetic measurements

For kinetic assays, NAD^+ (Boehringer Mannheim, grade I) was purified by the procedure of Dalziel (1963). NADH (Boehringer Mannheim, grade I) was purified by reverse-phase HPLC chromatography. NADH (4.2 mg) was injected on a RP-8 column (Brownlee Labs Aquapore Octyl C8.

0×250 mm) and eluted with a two-step linear salt gradient (200–100 mM Na_2SO_4 over 11.5 min and 100–0 mM Na_2SO_4 from 11.5 to 13.5 min) in sodium pyrophosphate buffer (1 mM, pH 7.0) at a flow rate of 2 ml/min. The collected NADH (3.6 mg, ~2 mM) had an absorption ratio of 2.26–2.27 (260/340 nm) and was essential free of sulfate ions. The purified NADH was directly used for the kinetic measurements. Steady state kinetic data were obtained in 32 mM Na pyrophosphate buffer (pH 8.2) containing Na sulfate (100 mM) at 25°C. The production and consumption of NADH was measured at 340 nm using an extinction coefficient of 6220 cm^{-1} M^{-1} (Horecker and Kornberg, 1948). Kinetic constants were derived from double reciprocal plots (Dalziel, 1975) for natural substrates and single reciprocal plots for long-chain alcohol substrates. The Haldane relationship with natural substrates gave an equilibrium constant of 14 pM. Substrate concentrations are provided in the captions to the tables. Errors are given as 90% confidence intervals.

Table I. Comparison of kinetic constants (at 25°C) for ethanol oxidation and acetaldehyde reduction by alcohol dehydrogenase (Adh 1) and the Trp54Leu mutant

	Adh 1[a]	Trp54Leu
k_{cat}^{Ox} (s^{-1})	308 ± 11	99.2 ± 1.4[b]
K_m^{NAD+} (μM)	211 ± 19	1090 ± 40[b]
$K_m^{ethanol}$ (mM)	4.03 ± 0.52	13.4 ± 0.6[c]
$k_{cat}/K_m^{ethanol}$ ($s^{-1}mM^{-1}$)	76.3 ± 7.1	7.64 ± 0.13
k_{cat}^{Red} (s^{-1})	932 ± 138	211 ± 10[d]
K_m^{NADH} (μM)	123 ± 28	94 ± 8[d]

[a]Concentrations of substrates were varied as follows: ethanol (4–20 mM) and NAD+ (0.2–2 mM) for ethanol oxidation and acetaldehyde (0.2–2.0 mM) and NADH (0.04–0.2 mM) for acetaldehyde reduction. In 32 mM sodium pyrophosphate buffer (pH 8.2) containing 100 mM Na_2SO_4 at 25°C.
[b]NAD^+ concentration was varied from 1 to 10 mM at fixed ethanol concentration (100 mM) in 32 mM sodium pyrophosphate buffer (pH 8.2) containing 100 mM Na_2SO_4 at 25°C.
[c]Ethanol concentration was varied between 5 and 50 mM at fixed NAD^+ concentration (10 mM) in 32 mM sodium pyrophosphate buffer (pH 8.2) containing 100 mM Na_2SO_4 at 25°C.
[d]NADH concentration was varied between 0.05 and 0.2 mM at fixed acetaldehyde concentration (20 mM) in 32 mM sodium pyrophosphate buffer (pH 8.2) containing 100 mM Na_2SO_4 at 25°C.

Results and discussion

Table I shows the kinetic parameters for ethanol oxidation and acetaldehyde reduction catalysed by wild type Adh 1 and the W54L mutant. Table II shows the kinetic parameter of native and mutant enzymes determined in parallel for several alcohols as substrates. With ethanol, 1-propanol and 1-butanol, the mutant performs less well than the native enzyme. However, with both 1-pentanol and 1-hexanol, the W54L mutant is the better catalyst. Diminishing the volume of the side chain at position 54 does not improve activity against secondary alcohols. These results are consistent with the model used to design these experiments, where the side chain of residue 54 contacts groups attached to the 4 position of long-chain primary alcohols rather than those nearer to carbon 1, where the oxidation takes place.

It is interesting to compare these results with those reported by Green *et al.* (1993), who replaced Trp54 in yeast Adh by a Met residue. Met was chosen as a replacement amino acid in their work because it is found at the homologous position in the human and rhesus monkey enzymes. Green *et al.* (1993) used slightly higher temperatures and different buffers in their kinetic measurements. Nevertheless, with native Adh I, the trends in k_{cat}/K_m obtained by Green *et al.* (1993) for different substrates are parallel to those obtained here (Table III). Both sets of data report consistent ratios for the activity of wild type Adh against various alcohol substrates, in particular, essentially identical increases in *V/K* upon going from 1-pentanol to 1-hexanol. Thus, there is good reason to believe that the data can be compared.

The most remarkable outcome of this comparison is the degree to which the results obtained upon substitution by Leu differ from those obtained by substitution by Met, especially considering the facility with which Leu and Met can be exchanged in other systems (see, for example, Stackhouse *et al.*, 1990) and during divergent evolution. Comparison ratios are presented in Table IV. Green *et al.* (1993) report that substitution of Trp54 by Met decreased both *V* and *V/K* for all of the alcohols examined. With 1-butanol and, better, 1-pentanol, the K_m values were decreased by the substitution, although not enough to make up for the decrease in *V*. Thus, for no substrate can it be said that replacement of Trp54 by a Met improves the catalytic performance of the enzyme.

Table II. Substrate specificity of alcohol dehyrogenase (Adh 1) and its Trp54Leu mutant with various alcohols[a]

	Wild type Adh 1				Mutant Trp54Leu			
	k_{cat}^{Ox} (s^{-1})	$K_m^{alcohol}$ (mM)	$k_{cat}/K_m^{alcohol}$ (s^{-1} mM^{-1})	k_{cat}/K_m (% of EtOH)	k_{cat}^{Ox} (s^{-1})	$K_m^{alcohol}$ (mM)	$k_{cat}/K_m^{alcohol}$ (s^{-1} mM^{-1})	k_{cat}/K_m (% of EtOH)
Primary alcohols								
Ethanol	308 ± 11	4.0 ± 0.5[b]	76.3 ± 7.1[b]	100	102.1 ± 2.8	13.4 ± 0.6	7.64 ± 0.13	100
Propanol	123 ± 3	13.6 ± 0.7	9.06 ± 0.14	11.9	28.7 ± 2.8	43.9 ± 5.5	0.65 ± 0.02	8.6
Butanol	118 ± 7	53.9 ± 4.3	2.20 ± 0.04	2.9	32.7 ± 1.8	29.4 ± 2.4	1.11 ± 0.03	14.5
Pentanol	56.2 ± 6.1	56.8 ± 7.6	0.99 ± 0.03	1.3	18.4 ± 0.5	11.8 ± 0.6	1.56 ± 0.04	20.4
Hexanol	29.6 ± 2.7	13.8 ± 2.0	2.14 ± 0.11	2.8	23.9 ± 1.8	6.5 ± 0.9	3.67 ± 0.22	48.0
3-Methyl-1-butanol	0.27[b]				0.08[b]			
4-Methyl-1-pentanol	6.7 ± 1.9	53.8 ± 17.9	0.115 ± 0.003	0.2	18.6 ± 0.7	6.7 ± 0.4	2.79 ± 0.05	36.5
Cinnamyl alcohol	133 ± 8	4.58 ± 0.35	29.0 ± 0.6	38.0	98.0 ± 3.9	1.5 ± 0.1	67.3 ± 2.9	881
Secondary alcohols								
2-Propanol	53.1 ± 10.1	268 ± 58	0.198 ± 0.005	0.3	5.8 ± 0.9	193 ± 33	0.030 ± 0.001	0.4
(S)-2-Butanol	11.9 ± 3.5	93.3 ± 33.9	0.127 ± 0.008	0.2	0.31[b]			

[a]Determined with 10 mM NAD^+ in sodium pyrophosphate buffer (32 mM, pH 8.2) containing 100 mM Na_2SO_4 at varying alcohol concentrations at 25°C.
[b]With an alcohol concentration of 50 mM.

Table III. Values of k_{cat}/K_m relative to ethanol for different substrates of alcohol dehydrogenase (Adh 1) and the Trp54Leu and Trp54Met mutants by independent kinetic measurements by Green *et al.* (1993) and in the present study

Substrate	Ethanol	1-Propanol	1-Butanol	1-Pentanol	1-Hexanol	Reference
Adh 1	100	22	4.7	3.9	8.5	Green *et al.* (1993)
W54M	100	5.2	6.8	9.0	1.4	Green *et al.* (1993)
Adh 1	100	11.9	2.9	1.3	2.8	Present study
W54L	100	8.6	14.5	20.4	48.0	Present study

All values are k_{cat}/K_m relative to ethanol (arbitrarily set to 100 for the indicated enzyme). Green *et al.* (1993): 83 mM potassium phosphate buffer, pH 7.3, 40 mM KCl, 0.25 mM EDTA and 10 mM NAD^+ at 30°C. Present study: 32 mM sodium pyrophosphate buffer, pH 8.2, 100 mM Na_2SO_4 and 10 mM NAD^+ at 25°C.

Table IV. Impact of the Trp54Leu and Trp54Met mutations on the V_{max} and K_m values of alcohol dehydrogenase

	Trp54Leu (present study)			Trp54Met [Green *et al.* (1993)]		
Alcohol substrate	V_{mut}/V_{wt}	$(K_m)_{mut}/(K_m)_{wt}$	$(V/K)_{mut}/(V/K)_{wt}$	V_{mut}/V_{wt}	$(K_m)_{mut}/(K_m)_{wt}$	$(V/K)_{mut}/(V/K)_{wt}$
Ethanol	0.33	3.35	0.10	0.65	2.65	0.25
1-Propanol	0.23	3.23	0.07	0.14	2.41	0.06
1-Butanol	0.28	0.55	0.50	0.27	0.75	0.37
1-Pentanol	0.33	0.21	1.58	0.20	0.35	0.58
1-Hexanol	0.81	0.47	1.72	0.47	1.60	0.28
4-Methyl-1-pentanol	2.78	0.12	24.3	ND	ND	ND
Cinnamyl alcohol	0.74	0.33	2.3	ND	ND	ND

V_{mut}, maximal velocity of mutant; V_w, maximal velocity of wild type; $(K_m)_{mut}$, Michaelis constant of mutant; $(K_m)_{wt}$, Michaelis constant of wild type.

These results are different with the W54L mutant. Here, the *V/K* values for 1-pentanol and 1-hexanol are increased relative to wild type. In both cases, the improved *V/K* arises predominantly because the K_m for the alcohol is lower in the mutant. However, for 1-hexanol, the k_{cat} of the mutant is essentially the same as in the native enzyme.

Although the structural model on which interpretations are based remains tentative, these results suggest that it is not sufficient simply to increase space in an active site to engineer a protein for a larger (hydrophobic) substrate. It appears as if some degree of precision must be attained in the steric complementarity between the hydrophobic substrate and the hydrophobic binding site. Similar conclusions might be drawn from experiments described in Ganzhorn *et al.* (1987), where Met294 (M270 in yeast) was replaced by Leu and in Weinhold *et al.* (1991), where Leu182 was replaced by Ala.

Results with branched chain primary alcohols are important for the use of the W54L mutant as a synthetic tool. With 3-methyl-1-butanol, the W54L mutant showed no catalytic superiority over native enzyme. With 4-methyl-1-pentanol, however, branched just one atom further along the chain, the mutant is considered better than native Adh. The k_{cat} is increased by a factor of three, the K_m decreased by a factor of eight and the *V/K* increased by a factor of 24. Cinnamyl alcohol is another substrate with branching at the 4 position. Here again, the *V/K* is improved by substituting Trp by Leu, although not as dramatically as with 4-methyl-1-pentanol. Data for 4-methyl-1-pentanol and cinnamyl alcohol as substrates were not reported for the W54M mutant (Green *et al.*, 1993); these would be interesting to have.

Perhaps the most interesting scientific result from this work is the extent to which the structural model for yeast Adh (Figure 1) built from the crystal structure of the homologous Adh from horse liver is serviceable as a tool for guiding mutagenesis experiments even as we move away from the reacting centre. Substituting Trp54 by Leu expands substrate specificities for exactly those molecules where it is expected: 1-pentanol, 1-hexanol, 4-methyl-1-pentanol and cinnamyl alcohol, where the model suggests that additional steric volume of the substrate will occupy the space previously filled by the side chain of Trp54.

When a crystal structure for yeast Adh 1 becomes available (Ramaswamy *et al.*, 1994), it will be interesting to learn whether the expected results occurred for the expected reasons. However, a simple generalization may be possible. Protein engineering experiments that involve alteration of substrate specificity via considerations of volume exclusion alone may generally produce results consistent with expectation, even in cases (such as this one) where the structural model of the active site is crude. In contrast, experiments involving alteration of complementarity in hydrogen bonding may be more challenging and demand models with higher overall resolution. Finally, alterations in charge complementarity may be still more challenging. It will be interesting to see how well future experiments conform to this generalization.

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