# Modeling substrate binding in *Thermus thermophilus* isopropylmalate dehydrogenase

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#### Abstract

The *Thermus thermophilus* 3-isopropylmalate dehydrogenase (IPMDH) and *Escherichia coli* isocitrate dehydrogenase (ICDH) are two functionally and evolutionarily related enzymes with distinct substrate specificities. To understand the determinants of substrate specificities of the two proteins, the substrate and coenzyme in IPMDH were docked into their respective binding sites based on the published structure for apo IPMDH and its sequence and structural homology to ICDH. This modeling study suggests that (1) the substrate and coenzyme (NAD) binding modes of IPMDH are significantly different from those of ICDH, (2) the interactions between the substrates and coenzymes help explain the differences in substrate specificities of IPMDH and ICDH, and (3) binding of the substrate and coenzyme should induce a conformational change in the structure of IPMDH.

Keywords: docking; isocitrate dehydrogenase; isopropylmalate dehydrogenase; substrate binding

Thermus thermophilus 3-isopropylmalate dehydrogenase (IPMDH) and Escherichia coli isocitrate dehydrogenase (ICDH) are two closely related enzymes that catalyze similar reactions in different metabolic pathways. IPMDH functions in the leucine biosynthesis pathway and catalyzes the oxidative decarboxylation of isopropylmalate to  $\alpha$ -ketoisocaproate (Imada et al., 1991). ICDH catalyzes a similar decarboxylation reaction in the carbohydrate metabolism (Kornberg, 1966), and converts isocitrate to  $\alpha$ -ketoglutarate. The two reactions are shown in Figure 1A. Both reactions are postulated to proceed in two steps, with dehydrogenation preceding decarboxylation (Fig. 1B) (Grissom & Cleland, 1985; Pirrung et al., 1994).

Most of the postulated key catalytic residues in *E. coli* ICDH (Hurley et al., 1991) are conserved in *T. thermophilus* IPMDH (Imada et al., 1991; Miyazaki et al., 1992). The two proteins have an overall 30% sequence identity (Fig. 2). The conservation of catalytic residues also extends to the IPMDHs and ICDHs of other species (Fig. 2), suggesting that these proteins may have a common evolutionary origin and structural, functional properties.

The substrate specificities of *T. thermophilus* IPMDH and *E. coli* ICDH are, however, very different. ICDH shows no detectable activities for isopropylmalate and IPMDH shows no activity for isocitrate (Miyazaki et al., 1993). A mammalian form of ICDH has also been extensively investigated (Colman, 1973;

Ehrlich & Colman, 1978). Miyazaki et al. (1993) showed that T. thermophilus IPMDH has broad substrate specificities against alkyl-malates. Substituting the  $\gamma$ -moiety of isopropylmalate with methyl, ethyl, or propyl groups has little effect on the overall catalytic efficiency  $(k_{cat}/K_m)$ , suggesting that the hydrophobic packing between the substrate and the enzyme-coenzyme complex is not very specific. On the other hand, IPMDH shows no detectable activity against isocitrate, suggesting that the polar or charge-charge interaction with the  $\gamma$ -carboxyl group destabilizes the IPMDH-substrate-coenzyme complex (Miyazaki et al., 1993). Studies on the roles of catalytic residues of IPMDH are limited. It was shown in a recent study that substituting Tyr 139 with phenylalanine resulted in a 10-fold decrease in the  $K_m$  of the coenzyme NAD but little effect on the  $K_m$  of the substrate (Miyazaki et al., 1993). The same mutation also resulted in a 10-fold decrease in the turnover number  $(k_{cat})$  and therefore did not change the overall catalytic activity at low substrate concentration. However, a similar change in the corresponding residue in ICDH (Tyr 160) resulted in a different change in the  $k_{cat}$  and  $K_m$  of isocitrate, suggesting that the two residues may play different roles in substrate binding and catalysis. Therefore, despite the sequence homology between IPMDH and ICDH, the two enzymes may have differences in their substrate binding and catalytic groups.

To interpret these distinct mechanisms and specificities, it is desirable to know the binding site of isopropylmalate to the IPMDH. To do so, the apo structure of IPMDH was aligned to the structure of ICDH based on the sequence homology (Fig. 2). This structural alignment reveals a general structural homology between the two proteins and suggested the possible

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 $+ CO_2$ 

Fig. 1. A: Reactions catalyzed by *T. ther-mophilus* IPMDH (Imada et al., 1991) and *E. coli* ICDH (Kornberg, 1966).  $X = C(CH_3)_2$  gives IPMDH and NAD<sup>+</sup>, and  $X = CH_2COO^-$  gives ICDH and NADP<sup>+</sup>. B: Both reactions are postulated to proceed in two steps, with dehydrogenation preceding decarboxylation (Grissom & Cleland, 1985; Pirrung et al., 1994).

substrate binding site of IPMDH. The isopropylmalate and NAD molecules were then docked to the protein surface of IPMDH by an automated docking method (Goodsell & Olson, 1990) to determine the possible substrate binding mode.

 $\begin{array}{ccc} cool & cool \\ c=0 & c=0 \\ c-c-H & H-c-H \\ c -H \end{array}$ 

#### **Results and discussion**

## Structural alignment of IPMDH and ICDH

To align structures of IPMDH and ICDH, we selected residues from each protein to define the transformation matrix that superimposes the two structures. The selection of these residues was based on (1) functional importance in the active sites and (2) evolutionary conservation among different species. The seven active site residues of *E. coli* ICDH, R129, R153, Y160, K230', D283', D307, and D311 were used (a prime, ', indicates the residue of the second subunit). The structural and functional importance of these residues was supported by their conservation in ICDHs from *E. coli* to mammals (Fig. 2). Sequence alignment of the ICDH and IPMDH showed the corresponding residues in IPMDH were: R104, R132, Y139, K185', D217', D241, and D245. These residues are also conserved in IPMDHs from different species (Fig. 2).

The atoms of the selected IPMDH residues were rigidly fitted to those corresponding atoms of ICDH by rotation and translation to minimize the RMS deviations between the coordinates of the two sets of atoms. The resulting transformation matrix was then used to align the IPMDH structure to that of ICDH. Views of the ICDH active site and the corresponding region of the aligned IPMDH are shown in Figure 3. The RMS deviation among the selected active site residues in the aligned structures is about 0.8 Å.

In previous studies, the two proteins were shown to have similar sequence and topology of secondary structures (Imada et al., 1991). The structural alignment shows that the tertiary structural homology between the two proteins is even more striking. Both proteins form functional dimers with the two-fold symmetry axes at the center of the dimer interfaces. Each dimeric complex contains two outside domains, one central domain, and two interdomain pockets. It was shown in the alignment that the two central domains from the two proteins were virtually superimposable. For example, the four-helix-bundles of the two proteins align well with each other at the dimer interface. So do other helices and  $\beta$ -strands in the central domains of the two proteins. The central and outside domains of the two proteins are connected by a group of  $\beta$ -strands, which also form the bottom of the interdomain pockets of the two proteins. These  $\beta$ -strands are superimposable in the two aligned structures. Although the two outside domains of the two proteins have similar structures, they were displaced from each other in the structural alignment. This displacement seems a result of a collective interdomain movement, such as a hinge motion. A tentative interdomain hinge motion in IPMDH may reduce the size of its interdomain pocket to that of ICDH's and, at the same time align its outside domain better with that of ICDH (Fig. 3A,B).

The structural alignment and homology suggest that the enzyme active site of IPMDH is located in its interdomain pocket. This is similar to ICDH, where the catalytic residues are located on the  $\alpha$ -helices and  $\beta$ -strands that form the sides and bottom of the interdomain pocket. The corresponding IPMDH residues are virtually superimposable on these ICDH catalytic residues (Fig. 3C). The side chains of R129 and 153 in ICDH, for example, took similar spatial positions as those of R104 and 132 in IPMDH. The aspartates in the active sites of IPMDH (D217',

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	1		20		40		60		
<pre>{A. tumefaciens }</pre>	mtvrsVflLP	GDGIGPEamt	evrKlieymn	sahnagF	tvsegLVGGs	AyDAhGva	isDadMEkal	aaDAILFGAV	GGPKWD
<pre>{B. caldotenax }</pre>	mgnyrIaVLP	GDGIGkEVts	gAVeVLKAVg	irfgheF	tFEygLIGGA	AIDeaGtP	LPEETvrlcr	esDAVLLGAV	GGPKWD
<pre>{B. coagulans }</pre>	.mkmklaVLP	GDGIGPEVmD	aAIrVLKtVl	Dndghea	vFEnaLIGGA	AIDeaGtP	LPEETLDicr	rsDAILLGAV	GGPKWD
(B. napus }	kkrynItlLP	GDGIGPEVis	iAknVLqqag	slegleF	sFqempVGGA	AlDlvGvP	LPEETvsAaK	esDAVLLGAI	GGyKWD
(B. subtilis )	.lkkr1alLP	GDGIGPEV1E	sAtdVLKsVa	ErfnheF	eFEygLIGGA	AIDehhnP	LPEETvaAcK	naDAILLGAV	GGPKWD
{C. maltosa }	vktkt1t1LP	GDhVGtEIvn	eAIKVLeAIe	aatpygkihF	dFkhhLIGGA	AIDATGvP	LPDDaLEsaK	nsDAVLLGAV	GGPKW
<pre>(C. pasteurianum)</pre>	mkefkIaViP	GDGIGPDIvr	eAVKImtkVg	EkydtkF	nFvevkaGGd	AIDAyGeP	LPkETiDvcK	ssaAVLLGAV	GGPKWD
{C. utilis }	mpektIvVLP	GDhVGtEIta	eAIKVLKAIe	EvkpeikF	nFqhhLIGGA	AIDATGvP	LPDDaLEAsK	kaDAVLLGAV	GGPKW
<pre>{K. lactis }</pre>	.mskn1vVLP	GDhVGkEVtD	eAIKVLnAIa	EvrpeikF	nFqhhLIGGA	AIDATGtP	LPDEaLEAsK	kaDAVLLGAV	GGPKW
<pre>{K. marxianus }</pre>	.mskn1vVLP	GDhVGtEItn	eAIKVLnAIs	EarpsikF	nFEhhLIGGA	AIDATGvP	LPDEAsK	kaDAVLLGAV	GGPKW
<pre>{L. interrogan }</pre>	mknVaVLs	GDGIGPEVmE	iAIsVLKkal	gakvseF	qFkegFVGGi	AIDkTGhP	LPpETLklce	essAILFGsV	GGPKWE
(S. platensis )	tqnyrItlLs	GDGIGPEIma	vAVdVLKAVg	kqldlnF	eFkeaLmGGv	AIDATGeP	LPEEsLqAcr	dsDAVLLaAI	GGyKWD
(S. pombe )	mcakkIvVLP	GDhIGPEIva	sAleVLKvVe	kkrpelkL	eFEehkIGGA	sIDAyGtP	LtDETvkAcl	eaDgVLLGAV	GGPeW
{S. tuberosum }	snliratlFP	GDGIGPEIaE	svrqIFKva.	evpi	eWEehYVGte	vdprTnsflt	WEsLEsvr	rnkvgLkGpm	atP
<pre>{T. aquaticus }</pre>	mrVaVLP	GDGIGPEVtE	aAlrVLKAld	EreglgL	tYEtfpfGGA	AIDgyGeP	FPEvTrkqve	aaEAVLLGsV	GGPKWD
<pre>{Y. lipolytica }</pre>	tdskkIvlLg	GDfcGPEVia	eAVKVLKsVa	Ea.sgteF	vFEdrLIGGA	AIEkeGeP	itDaTLDicr	kaDsIMLGAV	GGaantvwtt
{T. thermophilus}	mkVaVLP	GDGIGPEVtE	aAlKVLrAld	EaeglgL	aYEvfpfGGA	AIDAfGeP	FPEpTrkave	eaEAVLLGsV	GGPKWD
{pig mitochond. }	hyadqrikva	kpvVemDgdE	mtriIWqfIk	Eklilphvdv	qLkyfdlGlp	nrDqTndq	vtiDsalAtg	kysvavkcAt	itPd
{T. thermophilus}	ItViP	GDGIGPECVE	atlKVLeAa.	kapL	aYEvreaGas	vfrrgia.sg	vPqETiEsir	ktrvVLkGpl	etP
(S. cerevisiae )	laafskikvk	qpvVelDgdE	mtriIWdkIk	kklilpyldv	dLkyydlsve	srDATsdk	itqDaaEAiK	kygygikcAt	itPd
{S. cerevisiae }	ytvsfie	GDGIGPEIsk	svkKIFsAa.	nvpi	eWEscdVspi	fvngltt	iPDpavqsit	knlvaLkGpl	atP
{S. cerevisiae }	VtliP	GDGVGkEItD	svrtIFeAe.	nipi	dWEtinIkgt	dhkeq	.vyEavEslK	rnkigLkGlw	ht P
{ <b>E.</b> coli}	penpiIpyie	GDGIGvDVtp	amlKVvdAav	Ekaykgerki	sWmeiYtGek	stgvyGgdvw	LPaETLDlir	eyrvaikGpl	ttP
		-							
Consensus	I-VLP	GDGIGPEE	-AIKVLKA	EF	-FELIGGA	AIDATGP	LPDETLEA-K	DAVLLGAV	GGPKWD
	80		100		120		140		
<pre>(A. tumefaciens )</pre>	80 .gvpyehRPE	aG.LLrLRKD	100 L.eLFANL <b>R</b> P	aiCY.paLaa	120 aSsLKpElV.	eGlDilIV <b>R</b> E	140 LTGGV <b>Y</b> FGEp	kqiidlgng.	
<pre>(A. tumefaciens ) (B. caldotenax )</pre>	80 .gvpyehRPE .dnpph1RPE	aG.LLrLRKD kG.LLaiRKg	100 L.eLFANL <b>R</b> P L.dLYANL <b>R</b> P	aiCY.paLaa vvCY.dsLvs	120 aSsLKpElV. rSPLKpDlVq	eGlDilIV <b>R</b> E g.vDFVIV <b>R</b> E	140 LTGGV <b>Y</b> FGEp LTGGI <b>Y</b> FGqp	kqiidlgng. savveng.	
<pre>(A. tumefaciens ) {B. caldotenax } {B. coagulans }</pre>	80 .gvpyehRPE .dnpph1RPE .hnpas1RPE	aG.LLrLRKD kG.LLaiRKq kG.LLgLRKE	100 L.eLFANLRP L.dLYANLRP M.gLFANLRP	aiCY.paLaa vvCY.dsLvs vkaY.atLln	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe	eGlDilIV <b>RE</b> g.vDFVIV <b>RE</b> n.vDLVIV <b>R</b> E	140 LTGGV <b>Y</b> FGEp LTGGI <b>Y</b> FGqp LTGGI <b>Y</b> FGrp	kqiidlgng. savveng. serrgpg.	
<pre>(A. tumefaciens ) {B. caldotenax } {B. coagulans } {B. napus }</pre>	80 .gvpyehRPE .dnpphlRPE .hnpaslRPE .knekhlkPE	aG.LLrLRKD kG.LLaiRKq kG.LLgLRKE tG.LLgLRag	100 L.eLFANLRP L.dLYANLRP M.gLFANLRP L.kvFANLRP	aiCY.paLaa vvCY.dsLvs vkaY.atLln atvL.pgLvd	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aStLKrEva.	eGlDilIVRE g.vDFVIVRE n.vDLVIVRE eGvDLmVVRE	140 LTGGVYFGEp LTGGIYFGqp LTGGIYFGrp LTGGIYFGvp	kqiidlgng. savveng. serrgpg. rgiktneng.	
<pre>(A. tumefaciens ) (B. caldotenax ) {B. coagulans } (B. napus ) {B. subtilis }</pre>	80 .gvpyehRPE .dnpph1RPE .hnpas1RPE .knekh1kPE .gn1se1RPE	aG.LLrLRKD kG.LLaiRKq kG.LLgLRKE tG.LLqLRag kG.LLsiRKq	100 L.elfanl <b>r</b> L.dlyanl <b>r</b> M.glfanl <b>r</b> L.kvFanl <b>r</b> L.kvFanl <b>r</b> L.dlfanl <b>r</b>	aiCY.paLaa vvCY.dsLvs vkaY.atLln atvL.pqLvd vkvF.esLsd	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aStLKrEva. rSPLKkEyId	eGlDilIVRE g.vDFVIVRE n.vDLVIVRE eGvDLmVVRE n.vDFVIVRE	140 LTGGVYFGEp LTGGIYFGqp LTGGIYFGvp LTGGIYFGvp LTGGIYFGqp	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg.	
<pre>(A. tumefaciens ) (B. caldotenax ) {B. coagulans } (B. napus ) (B. subtilis ) (C. maltosa }</pre>	80 .gvpyehRPE .dnpph1RPE .hnpas1RPE .knekh1kPE .gn1se1RPE .gtga1RPE	aG.LLrLRKD kG.LLaiRKq kG.LLgLRKE tG.LLqLRag kG.LLsiRKq gG.LLkiRKE	100 L.elfanlrp L.dlyanlrp M.glfanlrp L.kvFanlrp L.dlfanlrp L.nlyanirp	aiCY.paLaa vvCY.dsLvs vkaY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aStLKrEva. rSPLKkEyId lSPLrpEvV.	eGlDilIVRE g.vDFVIVRE n.vDLVIVRE eGvDLMVVRE n.vDFVIVRE kGtnLIIVRE	140 LTGGVYFGEp LTGGIYFGqp LTGGIYFGvp LTGGIYFGqp LvGGIYFGp	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ee.geesed.	
<pre>(A. tumefaciens ) {B. caldotenax ) {B. coagulans } {B. napus } {B. subtilis } {C. maltosa } {C. pasteurianum}</pre>	80 .gvpyehRPE .dnpph1RPE .hnpas1RPE .knekh1kPE .gn1se1RPE .jgtga1RPE .nlegsRPE	aG.LLrLRKD kG.LLaiRKq kG.LLgLRKE tG.LLgLRAg kG.LLsiRKq qG.LLkiRKE ra.LLgLRga	100 L.eLFANLRP L.dLYANLRP M.gLFANLRP L.kvFANLRP L.dLFANLRP L.nLYANIRP L.gLYANLRP	aiCY.paLaa vvCY.dsLvs vkaY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle akvY.nvLks	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aStLKrEva. rSPLKkEyId lSPLrpEvV. aSPLKnEiId	eGlDilIVRE g.vDFVIVRE n.vDLVIVRE eGvDLmVVRE kGtnLIIVRE eGvDLIVVRE	140 LTGGVYFGEp LTGGIYFGQp LTGGIYFGVp LTGGIYFGVp LVGGIYFGDp LiGGIYFGD.	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ee.qeesed. rgtkev.ng.	
<pre>(A. tumefaciens ) {B. caldotenax } {B. coagulans } {B. napus } {B. subtilis } {C. maltosa } {C. pasteurianum} }</pre>	80 .gvpyehRPE .dnpphlRPE .hnpaslRPE .knekhlkPE .gtgalRPE .gtgalRPE .nlegskRPE .gtgaVRPE	aG.LLrLRKD kG.LLaiRKG kG.LLgLRKE tG.LLgLRAg kG.LLsiRKQ qG.LLkiRKE ra.LLgLRga qG.LLkiRKE	100 L.eLFANLRP M.gLFANLRP L.kvFANLRP L.kvFANLRP L.gLYANIRP L.gLYANLRP L.gLYANLRP	aiCY.paLaa vvCY.dsLvs vkaY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle akvY.nvLks cnFasesLld	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aStLKrEva. rSPLKkEyId lSPLFPEVV. aSPLKNEIId lSPiKaEvV.	eGlDillVRE g.vDFVIVRE eGvDLmVVRE n.vDFVIVRE kGtnLIIVRE kGtDLVVVRE	140 LTGGVYFGEp LTGGIYFGqp LTGGIYFGqp LTGGIYFGqp LTGGIYFGqp LVGGIYFGD. LVGGIYFGD.	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ee.qeesed. rgtkev.ng. ke.ddasg.	
<pre>(A. tumefaciens ) (B. caldotenax ) (B. coagulans ) (B. napus ) (B. subtilis ) (C. maltosa ) (C. pasteurianum) (C. utilis ) (K. lactis )</pre>	80 .gvpyehRPE .dnpph1RPE .hnpas1RPE .knekh1kPE .gtga1RPE .gtga1RPE .gtgavRPE .gtgavRPE	aG.LLrLRKD kG.LLaiRKq kG.LLgLRKE tG.LLgLRKE tG.LLsiRKq qG.LLkiRKE ra.LLgLRga qG.LLkiRKE gG.LLkiRKE	100 L.eLFANLRP M.gLFANLRP L.kVFANLRP L.kVFANLRP L.nLYANIRP L.gLYANLRP L.gLYANLRP L.gLYANLRP	aiCY.paLaa vvCY.dsLvs vkaY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle akvY.nvLks cnFasdsLld cnFasdsLld	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aStLKrEva. rSPLKkEyId lSPLrpEvV. aSPLKnEiId lSPLKAEVV. lSPLKDEVa.	eGlDilIVRE g.vDFVIVRE n.vDLVIVRE eGvDLmVVRE n.vDFVIVRE kGtnLIIVRE kGtDFVVVRE kGtDFVVVRE	140 LTGGVYFGEp LTGGIYFGqp LTGGIYFGqp LTGGIYFGp LVGGIYFGD LUGGIYFGD. LVGGIYFGD. LVGGIYFGET	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ee.qeesed. rgtkev.ng. ke.ddgsg ke.ddggg.	
<pre>(A. tumefaciens ) (B. caldotenax ) (B. coagulans ) (B. napus ) (B. subtilis ) (C. maltosa ) (C. mattosa ) (C. utilis ) (K. lactis ) (K. marxianus )</pre>	80 .gvpyehRPE .dnpphlRPE .hnpaslRPE .knekhlkPE .gtgalRPE .legskRPE .gtgavRPE .gtgavRPE .gtgavRPE	aG.LLrLRKD kG.LLgLRKE tG.LLgLRKE tG.LLgLRAg kG.LLsiRKq qG.LLkiRKE qG.LLkiRKE qG.LLkiRKE	100 L.eLFANLRP M.gLFANLRP L.dLFANLRP L.dLFANLRP L.nLYANIRP L.gLYANLRP L.gLYANLRP L.gLYANLRP L.gLYANLRP	aiCY.paLaa vvCY.dsLvs vkaY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle akvY.nvLks cnFasdsLld cnFasdsLld	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aStLKrEva. rSPLKkEyId lSPLrpEvV. aSPLKnEiId lSPiKaEvV. lSPLKpEya.	eGlDillVRE g.vDFVIVRE eGvDLmVVRE n.vDFVIVRE kGtnLIVRE kGtDFVVVRE kGtDFVVVRE kGtDFVVVRE	140 LTGGVYFGEp LTGGIYFGTp LTGGIYFGTp LTGGIYFGTp LVGGIYFGDT LVGGIYFGET LVGGIYFGET LVGGIYFGET	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ee.qeesed. rgtkev.ng. ke.ddgsg. ke.degdg.	
<pre>(A. tumefaciens ) (B. caldotenax ) (B. cagulans ) (B. napus ) (B. subtilis ) (C. maltosa ) (C. pasteurianum) (C. utilis ) (K. lactis ) (K. marxianus ) (L. interrogan )</pre>	80 .gvpyehRPE .hnpaslRPE .hnpaslRPE .knekhlkPE .gtgalRPE .nlegskRPE .gtgavRPE .gtgavRPE .gtgavRPE .gtgsvRPE .tlppekaPE	aG.LLrLRKD kG.LLaIRKG tG.LLqLRAg kG.LLsIRKG qG.LLkIRKG qG.LLkIRKE qG.LLkIRKE qG.LLKIRKE rGaLLDLRKh	100 L.eLFANLRP L.dLYANLRP L.kvFANLRP L.dLFANLRP L.dLFANLRP L.gLYANLRP L.gLYANLRP L.gLYANLRP F.dLFANLRP F.dLFANLRP	aiCY.paLaa vvCY.dsLvs vkaY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle cnFasdsLld cnFasdsLld cnFasdsLld aiY.peLkn	120 aSsLKpElV. rSPLKpDlVq aSPLKrEve aStLKrEva. rSPLKkEyId lSPLrpEvV. aSPLKnEiId lSPiKaEvV. lSPLKpEya. aSPvrsDiIg	eGlDillVRE g.vDFV1VRE eGvDLmVVRE n.vDFV1VRE kGtnL11VRE eGvDL1VVRE kGtDFVVVRE kGtDFVVVRE kGtDFVVVRE nGlDill1RE	140 LTGGVYFGEp LTGGIYFGTp LTGGIYFGTp LTGGIYFGTp LTGGIYFGTp LVGGIYFGET LVGGIYFGET LVGGIYFGET LTGGIYFGTp	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ee.qeesed. rgtkev.ng. ke.ddgsg. ke.degdg. ke.degdg. kgregsga.	
<pre>{A. tumefaciens } {B. caldotenax } {B. coagulans } {B. napus } {B. subtilis } {C. maltosa } {C. pasteurianum} {C. utilis } {K. lactis } {K. marxianus } {L. interrogan } </pre>	80 .gvpyehRPE .dnpph1RPE .hnpas1RPE .gnlse1RPE .gtga1RPE .gtga1RPE .gtgavRPE .gtgavRPE .gtgsvRPE .gtgsvRPE .lppeRPE	aG.LLrLRKD kG.LLaiRKq kG.LLgLRKE tG.LLgLRKE tG.LLsiRKq qG.LLkiRKE ra.LLgLRga qG.LLkiRKE qG.LLkiRKE rGaLLpLRKh tG.LLALRag	100 L.eLFANLRP M.gLFANLRP L.kVFANLRP L.kVFANLRP L.GLFANLRP L.GLYANLRP L.GLYANLRP L.GLYANLRP L.GLYANLRP F.dLFANLRP	aiCY.paLaa vvCY.dsLvs vkaY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle akvY.nvLks cnFasdsLld cnFasdsLld aiiY.peLkn llFc.phvld	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aStLKrEva ISPLKkEyId ISPLKpEv1. aSPLKnEiId ISPLKpEya. ISPLKpEya. aSPVrSDIIg aSsLKrEvV.	eGlDillVRE g.vDFVIVRE n.vDLVIVRE eGvDLMVVRE kGtnLIVRE kGtDFVVRE kGtDFVVRE kGtDFVVRE kGtDFVVRE cGDDILIRE eGvDimVRE	140 LTGGV¥FGEp LTGGI¥FGqp LTGGI¥FGvp LTGGI¥FGvp LTGGI¥FGD LVGGI¥FGD LVGGI¥FGEr LVGGI¥FGEr LTGGI¥FGEr LTGGI¥FGm	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ee.qeesed. rgtkev.ng. ke.ddggg. ke.ddggg. ke.degdg. kg.degdg. kgregsgq. kgregsgq.	
<pre>(A. tumefaciens ) (B. caldotenax ) (B. coagulans ) (B. napus ) (B. subtilis ) (C. maltosa ) (C. pasteurianum) (C. utilis ) (K. lactis ) (K. lactis ) (L. interrogan ) (S. platensis ) </pre>	80 .gvpyehRPE .dnpph1RPE .hnpas1RPE .knekh1kPE .gtga1RPE .gtga1RPE .gtgavRPE .gtgavRPE .gtgavRPE .gtgavRPE .tlppekqPE .hppeRPE	aG. LLrLRKD kG. LLgLRKE tG. LLgLRKE tG. LLgLRAg kG. LLsiRKq qG. LLkiRKE rG. LLkiRKE rGaLLpLRKh tG. LLkIRKE rGaLLpLRKs	100 L. eLFANLRP L. dLYANLRP L. dLYANLRP L. ALFANLRP L. ALYANLRP L. ALYANLRP L. GLYANLRP L. GLYANLRP F. dLFANLRP F. dLFANLRP M. GYFANLRP	aiCY.paLaa vvCY.dsLvs vkAY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle cnFasdsLld cnFasdsLld aiiY.peLkn llFc.phvld cnFasksLvk	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aStLKrEva. rSPLKkEyId lSPLKpEVJ. aSPLKpEya. aSPVrsDiIg aSsLKrEvV. ySPLKoEiV.	eGlDillVRE g.vDFVIVRE n.vDFVIVRE eGVDLMVVRE kGtnLIVVRE kGtDFVVVRE kGtDFVVVRE kGtDFVVVRE nGlDillRE eGVDIMVVRE eGVDFCVVRE	140 LTGGVYFGEp LTGGIYFGUp LTGGIYFGUp LTGGIYFGUp LVGGIYFGDr LVGGIYFGEr LVGGIYFGEr LVGGIYFGEP LTGGIYFGQp LTGGIYFGEP	kqiidlgng. savveng. rgjktneng. skryvnteg. ee.qeesed. rgtkev.ng. ke.ddgsg. ke.degdg. ke.degdg. kgregsgq. kgifetetg.	
<pre>(A. tumefaciens ) (B. caldotenax ) (B. coagulans ) (B. subtilis ) (C. maltosa ) (C. maltosa ) (C. utilis ) (K. lactis ) (K. marxianus ) (L. interrogan ) (S. pombe ) (S. tuberosum )</pre>	80 .gvpyehRPE .hnpaslRPE .knekhlkPE .gnlselRPE .gtgaRPE .gtgavRPE .gtgavRPE .gtgsvRPE .lppekqPE .lppekqPE iqk.gh	aG.LLrLRKD KG.LLaIRKQ KG.LLQLRKE KG.LLQLRAg kG.LLSIRKQ qG.LLkIRKE qG.LLKIRKE qG.LLKIRKE qG.LLKIRKE tGLLDLRKh tG.LLALRAg qG.LLKLRKE	100 L. eLFANLRP L. dLYANLRP L. kUFANLRP L. dLFANLRP L. dLFANLRP L. gLYANLRP L. gLYANLRP L. gLYANLRP F. dLFANLRP F. dLFANLRP L. gLYANLRP L. gLYANLRP L. gLYANLRP L. gLYANLRP L. gLYANLRP L. gLYANLRP	aiCY.paLaa vvCY.dsLvs vkaY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLld cnFasdsLld cnFasdsLld aiiY.peLkn llFc.phvld cnFasksLvk vysLpgvktr	120 aSsLKpElV. rSPLKpDlVq aSPLKrEvva. rSPLKkEyId lSPLrpEvV. aSPLKnEiId lSPiKaEvV. lSPLKpEya. aSPvrsDiIg aSsLKrEvV. ySPLKpEiV. vd	eGlDillVRE g.vDFV1VRE eGvDLmVVRE n.vDFV1VRE kGtnL11VRE kGtDFVVVRE kGtDFVVVRE kGtDFVVVRE nGlDill1RE eGvDimVVRE eGvDFcVVRE dV.nL11RE	140 LTGGVYFGEp LTGGIYFGTp LTGGIYFGTp LTGGIYFGTp LTGGIYFGD LVGGIYFGET LVGGIYFGET LTGGIYFGET LTGGIYFGTp LTGGIYFGTp LTGGIYFGTp LTGGIYFGTp	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ee.qeesed. rgtkev.ng. ke.ddgsg. ke.degdg. kg.degdg. kgregsgq. kgifetetg. te.dngsgy.	
<pre>(A. tumefaciens ) (B. caldotenax ) (B. napus ) (B. subtilis ) (C. maltosa ) (C. pasteurianum) (C. utilis ) (K. lactis ) (K. marxianus ) (L. interrogan ) (S. platensis ) (S. pombe ) (S. tuberosum ) (T. aquaticus )</pre>	80 .gvpyehRPE .dnpph1RPe .hnpas1RPE .gtga1RPE .gtga1RPE .gtga1RPE .gtgaVRPE .gtgsVRPE .gtgsVRPE .lppeRPE .lppeRPE igk.gh .alprkiRPE	aG.LLrLRKD kG.LLaiRKq kG.LLgLRKE tG.LLgLRKE tG.LLkIRKG qG.LLkiRKE ra.LLgLRga qG.LLkiRKE qG.LLkiRKE qG.LLkIRKE rGaLLpLRKh tG.LLaLRag qG.LLkLRKS rslnLtLRKE	100 L. eLFANLRP M. gLFANLRP L. &LYANLRP L. &LYANLRP L. GLYANLRP L. GLYANLRP L. GLYANLRP L. GLYANLRP L. GLYANLRP L. GLYANLRP L. GLFANTAT M. GVWANLRP L. NLYANVRP g. dLFANLRP	aiCY.paLaa vvCY.dsLvs vkaY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle akvY.nvLks cnFasdsLld cnFasdsLld aiiY.peLkn llFc.phvld cnFasksLvk cysLpgyktr akvF.pgLer	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aStLKrEva. rSPLKkEyId lSPLrpEvV. aSPLKnEiId lSPLKpEya. lSPLKpEya. aSPVrSDiIg aSsLKrEvV. ySPLKpEiV. yd	eGlDillVRE g.vDFVIVRE n.vDLVIVRE eGvDLMVVRE kGtnLIVRE kGtDFVVVRE kGtDFVVVRE kGtDFVVVRE cGvDDillRE eGvDimVVRE dv.nLILRE rGvDFLVRE	140 LTGGV¥FGEp LTGGI¥FGqD LTGGI¥FGvp LTGGI¥FGvp LTGGI¥FGD LVGGI¥FGD LVGGI¥FGEr LVGGI¥FGEr LTGGI¥FGEp LTGGI¥FGQp LTGGC¥FGEr nTEGE¥SGIe	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ke.ddgsg. ke.ddgsg. ke.degdg. ke.degdg. kgregsgq. kgregsgq. tgitetg. te.dngsgy. hqvvrgvve.	
<pre>(A. tumefaciens ) (B. caldotenax ) (B. coagulans ) (B. subtilis ) (C. maltosa ) (C. pasteurianum) (C. utilis ) (K. lactis ) (L. interrogan ) (S. platensis ) (S. pombe ) (S. tuberosum ) (T. aquaticus )</pre>	80 .gvpyehRPE .dnpph1RPE .hnpas1RPE .knekh1kPE .gtga1RPE .gtga1RPE .gtgavRPE .gtgavRPE .gtgsvRPE .tlppekqPE .tlppekqPE .tlppeRpE .tlppcRPE .tlppcRPE .tlppcRPE .tlppcRPE .tlppcRPE	aG.LLrLRKD kG.LLgLRKE tG.LLgLRKE tG.LLgLRAg kG.LLsiRKq qG.LLkiRKE rg.LLkiRKE rGaLLpLRKh tG.LLkIRKE rGaLLpLRKh tG.LLALRAg gG.LLkLRKS rSlnLtLRKE sG.LLALRKS	100 L. eLFANLRP L. dLYANLRP L. dLYANLRP L. NLYANLRP L. NLYANLRP L. JLYANLRP L. GLYANLRP L. GLYANLRP F. dLFANLRP F. dLFANLRP L. GLFANTAT M. GVWANLRP L. NLYANVRP Q. dLFANLRP	aiCY.paLaa vvCY.dsLvs vkAY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle akvY.nvLks cnFasdsLld cnFasdsLld aiiY.peLkn llFc.phvld cnFasdsLvk cysLpgyktr akvF.pgLer cdlLspkLad	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aStLKrEva. rSPLKkEyId lSPLKpEVJ. aSPLKpEya. aSPVrsDIIg aSsLKrEvV. ySPLKpEiV. ySPLKpEiV. JSPLKEE.Ia lSPLKE.IA	eGlDillVRE g.vDFVIVRE n.vDFVIVRE eGVDLMVVRE kGtnLIVVRE kGtDFVVVRE kGtDFVVVRE kGtDFVVVRE eGVDFVVVRE eGVDFCVVRE dv.nLfIRE rGvDvlIVRE eGtDFIVRE	140 LTGGVYFGEp LTGGIYFGUp LTGGIYFGUp LTGGIYFGUp LVGGIYFGDr LVGGIYFGEr LVGGIYFGEr LVGGIYFGEr LTGGIYFGQp LTGGIYFGQp LTGGIYFGQp LTGGIYFGEr nTeGeYSGIe LTGGIYFGEr	kqiidlgng. savveng. rgjktneng. skryvnteg. ee.qeesed. rgtkev.ng. ke.ddgsg. ke.degdg. ke.degdg. kgregsgq. kgifetetg. kgifetetg. hqvvrgvve. rgms. ke.daga.	
<pre>(A. tumefaciens ) (B. caldotenax ) (B. coagulans ) (B. subtilis ) (C. maltosa ) (C. maltosa ) (C. utilis ) (K. lactis ) (K. lactis ) (L. interrogan ) (S. platensis ) (S. pombe ) (S. tuberosum ) (T. aquaticus ) (Y. lipolytica )</pre>	80 .gvpyehRPE .dnpphlRPE .hnpaslRPE .knekhlkPE .gtgalRPE .gtgaVRPE .gtgavRPE .gtgavRPE .tlppekqPE .nlprpeRPE .tlppekqPE .lgrgavRPE .lgrgavRPE .lgrgavRPE .lgrgavRPE .lprpeRPE .lgrgkvRPE .lgrkiRPE gdgrtdvRPE	aG.LLrLRKD kG.LLaiRKq kG.LLgLRKE tG.LLqLRag kG.LLsiRKq qG.LLkiRKE qG.LLkiRKE qG.LLkiRKE qG.LLkiRKE tG.LLaLRag qG.LLkLRKS rslnLtLRKE sG.LLsLRKS	100 L. eLFANLRP M. gLFANLRP L. dLYANLRP L. dLFANLRP L. dLFANLRP L. gLYANLRP L. gLYANLRP L. gLYANLRP F. dLFANLRP F. dLFANLRP G. dLFANLRP g. dLFANLRP g. dLFANLRP	aiCY.paLaa vvCY.dsLvs vkaY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle akvY.nvLks cnFasdsLld cnFasdsLld aiiY.peLkn llFc.phvld cnFasksLvk cysLpgyktr akvF.pgLer cqlLspkLad akvF.pgLer	120 aSsLKpElV. rSPLKpDlVq aSPLKrEvva. rSPLKkEyId lSPLrpEvV. aSPLKpEya. aSPLKpEya. aSPvrSDiIg aSsLKrEvV. ySPLKpEiV. ySPLKpEiV. yd lSPLKeE.Ia	eGlDillVRE g.vDFV1VRE eGvDLmVVRE n.vDFV1VRE kGtnL1VVRE kGtDFVVVRE kGtDFVVVRE nGlDillRE eGvDFVVVRE eGvDFVVRE gVDFCVVRE gTDFUVRE gTDF1VRE eGtDF1VRE	140 LTGGVYFGEp LTGGIYFGTp LTGGIYFGTp LTGGIYFGTp LVGGIYFGDr LVGGIYFGEr LVGGIYFGEr LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. e.qeesed. rgtkev.ng. ke.ddgsg. ke.degdg. kgregsgq. kgifetetg. te.dngsgy. te.dngsgy. rgms. ke.ddgsg.	
<pre>(A. tumefaciens ) (B. caldotenax ) (B. napus ) (B. subtilis ) (C. maltosa ) (C. pasteurianum) (C. utilis ) (C. utilis ) (K. lactis ) (K. marxianus ) (L. interrogan ) (S. platensis ) (S. pombe ) (S. tuberosum ) (T. aquaticus ) (Y. lipolytica ) (T. thermophilus)</pre>	80 .gvpyehRPE .dnpph1RPE .hnpas1RPE .gtga1RPE .gtga1RPE .gtga1RPE .gtgavRPE .gtgavRPE .gtgsvRPE .gtgsvRPE .lppekqPE .nlprpeRPE .lppekqPE .lprkRPE .gtg.dvRPE .gtg.dvRPE .gtg.dvRPE .lprkRPE .lprkRPE .gtgrtdvRPE .gtprkiRPE	aG.LLrLRKD kG.LLaiRKq kG.LLgLRKE tG.LLgLRKE tG.LLgLRKG qG.LLkiRKE ra.LLgLRga qG.LLkiRKE qG.LLkiRKE qG.LLkiRKE rGaLLpLRKh tG.LLaLRag qG.LLkLRKS rSlnLtLRKE gG.LL&LRKS tG.LLSLRKS	100 L. eLFANLRP M. gLFANLRP L. ALYANLRP L. ALYANLRP L. GLYANLRP L. GLYANLRP L. GLYANLRP F. GLFANLRP L. GLYANLRP L. GLFANTAT M. GVWANLRP L. ALYANLRP Q. GLFANLRP L. ALYANLRP Q. GLFANLRP	aiCY.paLaa vvCY.dsLvs vkaY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle akvY.nvLks cnFasdsLld cnFasdsLld aiiY.peLkn llFc.phvld cnFasksLvk cysLpgyktr cqlLspkLad akvF.pgLer	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aStLKrEve aSPLKREYId lSPLKpEyd lSPLKpEya. lSPLKpEya. aSPVrSDiIg aSsLKrEvV. ySPLKpEiV. yd lSPLKEE.Ia lSPIrn.V. lSPLKEE.Ia	eGlDillVRE g.vDFVIVRE n.vDLVIVRE eGVDLMVVRE kGtnLIVRE kGtDFVVVRE kGtDFVVVRE kGtDFVVVRE cGVDILIRE eGVDIMVRE dV.nLILRE eGVDFVVRE rGVDVIVRE rGVDVIVRE	140 LTGGV¥FGEp LTGGI¥FGqD LTGGI¥FGqD LTGGI¥FGqD LTGGI¥FGQD LVGGI¥FGGT LVGGI¥FGET LVGGI¥FGET LTGGI¥FGED LTGGI¥FGED LTGGI¥FGED LTGGI¥FGED LTGGI¥FGED LTGGI¥FGED	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ke.ddgsg. ke.ddgsg. ke.degdg. kgregsgq. kgrifetetg. te.dngsgy. hqvvrgvve. rgms. ke.ddgsg. rgms.	
<pre>(A. tumefaciens ) (B. caldotenax ) (B. cagulans ) (B. napus ) (B. subtilis ) (C. maltosa ) (C. pasteurianum) (C. utilis ) (K. lactis ) (K. marxianus ) (L. interrogan ) (S. platensis ) (S. pubee ) (S. tuberosum ) (T. aquaticus ) (Y. lipolytica ) (T. thermophilus) </pre>	80 .gvpyehRPE .dnpph1RPE .hnpas1RPE .knekh1kPE .gtgatRPE .gtgatRPE .gtgatRPE .gtgatRPE .gtgstRPE .lppekqPE .lppeRPE igk.gh .alprkiRPE .glprkiRPE 	aG.LLrLRKD kG.LLgLRKE tG.LLgLRKE tG.LLgLRKE tG.LLgLRKG qG.LLkiRKE ra.LLgLRga qG.LLkiRKE rGaLLpLRKh tG.LLkIRKE rGaLLpLRKh tG.LLkLRKS rsinLtLRKE sG.LLkLRKS tG.LLkLRKS	100 L. eLFANLRP M. gLFANLRP L. dLYANLRP L. dLFANLRP L. dLFANLRP L. gLYANLRP L. gLYANLRP L. gLYANLRP L. gLYANLRP L. gLYANLRP G. dLFANLRP g. dLFANLRP g. dLFANLRP g. dLFANLRP g. dLFANLRP	aiCY.paLaa vvCY.dsLvs vkAY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle akvY.nvLks cnFasdsLld cnFasdsLld aiiY.peLkn llFc.phvld cnFasksLvk cysLpgyktr akvF.pgLer cqLLspkLad akvF.pgLer gtvFrepii.	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aSrLKrEva. rSPLKkEyId lSPLrpEvV. aSPLKnEiId lSPLKpEya. lSPLKpEya. aSPVrsDiIg aSsLKrEvV. ySPLKpEiV. ySPLKpEIV. jSPLKeE.Ia lSPirn.V. lSPLKeE.Ia ckniprlvpg	eGlDillVRE g.vDFVIVRE n.vDFVIVRE eGVDLMVVRE kGtDILVVRE kGtDFVVVRE kGtDFVVVRE kGtDFVVVRE gGVDILIRE eGVDMVVRE gGVDTVVRE rGVDVIVRE rGVDVIVRE rGVDVIVRE rGVDVIVRE	140 LTGGVYFGEp LTGGIYFGUp LTGGIYFGUp LTGGIYFGUp LTGGIYFGD LUGGIYFGD LUGGIYFGD LUGGIYFGET LUGGIYFGET LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP LUGGIYFGEP LUGGIYFGEP AbGdqYkatd	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ee.qeesed. rgtkev.ng. ke.ddggg. ke.degdg. kg.degdg. kgifetetg. te.dngsgy. hqvvrgvve. rgms. ke.ddgsg. rgms.	ivftpkdgss
<pre>(A. tumefaciens ) (B. caldotenax ) (B. coagulans ) (B. subtilis ) (C. maltosa ) (C. pasteurianum) (C. utilis ) (K. lactis ) (L. interrogan ) (S. platensis ) (S. pube ) (S. tuberosum ) (T. aquaticus ) (T. thermophilus) </pre>	80 .gvpyehRPE .dnpph1RPE .hnpas1RPE .knekh1kPE .gtga1RPE .gtgavRPE .gtgavRPE .gtgsvRPE .tlppekqPE .tlppekqPE .tlppekqPE .tlppekqPE .tlppekqPE .gtgrtdvRPE .gdprtdvRPE .glprtkIRPE	aG. LLrLRKD kG. LLgLRKE tG. LLgLRKE tG. LLgLRKE tG. LLgLRAg kG. LLsIRKQ qG. LLkiRKE qG. LLkiRKE qG. LLkIRKE rGaLLpLRKh tG. LLaLRAS qG. LLkLRKS rSlnLtLRKE sG. LLALRKS tG. LLSLRKS efkLkkMWKS	100 L. eLFANLRP L. dLYANLRP L. dLYANLRP L. ALFANLRP L. ALFANLRP L. ALYANLRP L. GLYANLRP L. GLYANLRP L. GLYANLRP F. dLFANLRP G. dLFANLRP L. ALYANVRP q. dLFANLRP p. dLFANLRP pngtirNilg	aiCY.paLaa vvCY.dsLvs vkaY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle akvY.nvLks cnFasdsLld cnFasdsLld aiiY.peLkn llFc.phvld cnFasdsLvk cysLpgyktr akvF.pgLer cqlLspkLad akvF.pgLer gtvFrepii. gtvFrepiv.	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aStLKrEva. rSPLKkEyId lSPLKpEyu. aSPLKpEya. aSPVrsDiIg aSsLKrEvV. ySPLKpEiv. ySPLKpEiv. ySPLKeE.Ia lSPirn.V. lSPLKeE.Ia ckniprlvpg ipriprlvpr	eGlDillVRE g.vDFVIVRE n.vDLVIVRE eGvDLmVVRE kGtnLIVVRE kGtDFVVVRE kGtDFVVVRE kGtDFVVVRE nGlDillRE eGvDFcVVRE dv.nLILRE rGvDvlIVRE eGtDFIVRE eGtDFIVRE wtkpitIgRh wekpitIgRh	140 LTGGVYFGEp LTGGIYFGTp LTGGIYFGTp LTGGIYFGTp LTGGIYFGDp LVGGIYFGDr LVGGIYFGEr LVGGIYFGEr LTGGIYFGTp LTGGIYFGTp LTGGIYFGEp LTGGIYFGEp LTGGIYFGEp LTGGIYFGEp LTGGIYFGEp LTGGIYFGEp	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ee.qeesed. rgtkev.ng. ke.ddggg. ke.degdg. kgregsgq. kgifetetg. kgifetetg. ke.ddgsg. rgms. ke.ddgsg. rgms. fvvdragtfk	ivftpkdgss lvykpsdptt
<pre>(A. tumefaciens ) (B. caldotenax ) (B. napus ) (B. subtilis ) (C. maltosa ) (C. pasteurianum) (C. utilis ) (C. utilis ) (K. marxianus ) (L. interrogan ) (S. platensis ) (S. pombe ) (S. tuberosum ) (T. aquaticus ) (Y. lipolytica ) (T. thermophilus) (S. crevisiae )</pre>	80 .gvpyehRPE .dnpph1RPE .hnpas1RPE .gtga1RPE .gtga1RPE .gtga1RPE .gtgavRPE .gtgsvRPE .tlppekqPE .lppekqPE .lppekqPE .lprkiRPE pdgrtdvRPE .glprkiRPE .glprkiRPE eaRvk eaRvk	aG.LLrLRKD KG.LLaiRKQ kG.LLgLRKE tG.LLgLRKE tG.LLgLRKG ra.LLgLRKG ra.LLgLRKA rgG.LLkiRKE rGaLLpLRKA tG.LLaLRAg rG.LLkLRKS rslnLtLRKE sG.LLaLRKS rgG.LLSLRKS efnLhKMWKS rslnLtLRKE	100 L.eLFANLRP M.gLFANLRP L.kVFANLRP L.kVFANLRP L.dLFANLRP L.gLYANLRP L.gLYANLRP L.gLYANLRP F.GLFANLRP L.gLYANLRP L.gLFANTAT M.gVWANLRP Q.dLFANLRP Q.dLFANLRP Q.dLFANLRP G.GLFANLRP F.GLFANVRP	aiCY.paLaa vvCY.dsLvs vkaY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle akvY.nvLks cnFasdsLld cnFasdsLld aiiY.peLkn llFc.phvld cnFasksLvk cysLpgykt akvF.pgLer cqlLspkLad akvF.pgLer gtvFrepiv. aksigfktt	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aStLKrEve aSPLKREYId lSPLKpEyid lSPLKpEya. lSPLKpEya. aSPVrsDiIg aSsLKrEvV. ySPLKpEiV. yd lSPLKeE.Ia lSPIrn.V. lSPLKeE.Ia ckniprlvpg ipriprlvpr	eGlDillVRE g.vDFVIVRE n.vDFVIVRE n.vDFVIVRE kGtnLIVRE kGtDFVVVRE kGtDFVVVRE kGtDFVVVRE dV.nLILRE eGvDimVVRE eGvDFVVRE dv.nLILRE rGvDvlIVRE wtkpitIgRh wv.bLVIRE	140 LTGGVYFGEp LTGGIYFGqp LTGGIYFGp LTGGIYFGp LTGGIYFGD LGGIYFGD LGGIYFGD LGGIYFGET LGGIYFGT LTGGIYFGT LTGGIYFGT LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP LGGIYFGEP LGGIYFGEP LGGIYFGEP LGGIYFGEP LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ke.degdg. ke.ddggg. ke.degdg. kgregsgq. kgifetetg. te.dngsgy. hqvvrgvve. rgms. ke.ddgsg. rgms. ke.ddgsg. fvvdragtfk tlipgpgsle hivcpgvva.	ivftpkdgss lvykpsdptt
<pre>(A. tumefaciens ) (B. caldotenax ) (B. coagulans ) (B. napus ) (B. subtilis ) (C. maltosa ) (C. pasteurianum) (C. utilis ) (K. lactis ) (K. marxianus ) (L. interrogan ) (S. pombe ) (S. tuberosum ) (S. tuberosum ) (T. aquaticus ) (Y. lipolytica ) (T. thermophilus) (S. cerevisiae ) (S. cerevisiae )</pre>	80 .gvpyehRPE .dnpph1RPE .hnpas1RPE .knekh1kPE .gtgalRPE .gtgaVRPE .gtgaVRPE .lppekqPE .lppekqPE .lprpeRPE igk.gh .alprkiRPE pdgrtdvRPE .glpkiRPE .dlprkiRPE eaRvk 	aG.LLrLRKD kG.LLaiRKq kG.LLgLRKE tG.LLgLRKE tG.LLsIRKQ qG.LLkiRKE qG.LLkiRKE qG.LLkiRKE qG.LLkiRKE rGaLLPRKh tG.LLaLRag qG.LLkLRKS rsInLtLRKE sG.LLALRKS qG.LLkLRKS rsInLtLRKS rsInLtKMWKS efnLhKMWKS	100 L.eLFANLRP M.gLFANLRP L.dLYANLRP L.kVFANLRP L.dLFANLRP L.gLYANLRP L.gLYANLRP L.gLYANLRP L.gLYANLRP L.gLYANLRP G.dLFANLRP Q.dLFANLRP Q.dLFANLRP Q.dLFANLRP Q.dLFANLRP L.nLYANLRP Q.dLFANLRP L.nLYANLRP Q.dLFANLRP L.GLFANLRP L.GLFANLRP L.GLFANLRP L.GLFANLRP L.GLFANLRP L.GLFANLRP	aiCY.paLaa vvCY.dsLvs vkAY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle akvY.nvLks cnFasdsLld cnFasdsLld aiiY.peLkn llFc.phvld cnFasksLvk cysLpgyktr akvF.pgLer cqlLspkLad akvF.pgLer gtvFrepii. gtvFrepii. aksiegfktt fksLkgvktr	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aSrLKrEve aSPLKkEyId lSPLrpEvV. aSPLKpEya. lSPLKpEya. aSPVrsDiIg aSsLKrEvV. ySPLKpEiV. ySPLKpEIV. lSPLKeE.Ia lSPirn.V. lSPLKeE.Ia ckniprlvpg ipriprlvpr ye ip	eGlDilIVRE g.vDFVIVRE n.vDFVIVRE eGVDLMVVRE kGtDIIVRE kGtDFVVVRE kGtDFVVVRE kGtDFVVVRE kGtDFVVVRE cGVDFCVVRE dV.nLIIRE cGVDFIVRE cGVDVIVRE cGVDVIVRE rGVDVIVRE rGVDVIVRE mkpiIIGRh wekpiIIGRh v.DLVIRE	140 LTGGVYFGEp LTGGIYFGTp LTGGIYFGTp LTGGIYFGTp LTGGIYFGDT LVGGIYFGDT LVGGIYFGET LVGGIYFGET LTGGIYFGET LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP LVGGIYFGEP LVGGIYFGEP LVGGIYFGEP TGGIYFGEP TGGIYFGEP TGGIYFGEP TGGIYFGEP TGGIYFGEP	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ee.qeesed. rgtkev.ng. ke.ddggg. ke.degdg. kg.degdg. kgregsgq. kgifetetg. te.dngsgy. hqvvrgvve. rgms. ke.ddgsg. rgms. ke.ddgsg. rgms. fvvdragtfk tlipgpgsle hesvpgvve.	ivftpkdgss lvykpsdptt
<pre>(A. tumefaciens ) (B. caldotenax ) (B. coagulans ) (B. napus ) (B. subtilis ) (C. maltosa ) (C. pasteurianum) (C. utilis ) (K. lactis ) (K. lactis ) (K. natrianus ) (L. interrogan ) (S. platensis ) (S. pombe ) (S. tuberosum ) (T. aquaticus ) (T. thermophilus) (S. cerevisiae ) </pre>	80 .gvpyehRPE .dnpph1RPE .hnpas1RPE .knekh1kPE .gtga1RPE .gtga1RPE .gtgavRPE .gtgsvRPE .tlppekqPE .tlppekqPE .tlppeRPE .dtgrkIRPE .glprkIRPE .glprkIRPE eaRvk eaRvk 	aG.LLrLRKD kG.LLgLRKE tG.LLgLRKE tG.LLgLRKE tG.LLgLRAg kG.LLsiRKG qG.LLkiRKE rG.LLkiRKE rGaLLLkIRKE rGaLLLKIRKS rSlnLtLRKS sG.LL&LRKS cG.LL&LRKS efkLkKMWKS rSlnLtLRKE sslnLtLRKE sslnLtRKE ksanvtLRK1	100 L. eLFANLRP L. dLYANLRP L. dLYANLRP L. kvFANLRP L. dLFANLRP L. gLYANLRP L. gLYANLRP L. gLYANLRP F. dLFANLRP F. dLFANLRP G. dLFANLRP g. dLFANLRP g. dLFANLRP g. dLFANLRP pngtirNilg F. gLFANVRP L. gLFANVRP	aiCY.paLaa vvCY.dsLvs vkAY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle akvY.nvLks cnFasdsLld cnFasdsLld aiiY.peLkn llFc.phvld cnFasdsLvk cysLpgyktr akvF.pgLer cqlLspkLad akvF.pgLer gtvFrepii. gtvFrepiv. aksiegfktt fksLkgvktr	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aStLKrEva. rSPLKkEyId lSPLKpEyu. aSPLKpEya. aSPVrSDIIg aSsLKrEvV. ySPLKpEiv. yG lSPLKeE.Ia lSPLKeE.Ia ckniprlvpg ipriprlvpr ye ip	eGlDillVRE g.vDFVIVRE n.vDFVIVRE eGVDLMVVRE kGtDILVVRE kGtDFVVVRE kGtDFVVVRE kGtDFVVVRE cGVDFVVVRE dV.nLILIRE rGVDV11VRE eGVDFLIVRE wtkpitIgRh wv.DLV1IRE di.DLVVIRE	140 LTGGVYFGEp LTGGIYFGUp LTGGIYFGUp LTGGIYFGUp LTGGIYFGDr LVGGIYFGDr LVGGIYFGEr LVGGIYFGEr LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP AhGdqYkatd ahGdqYkatd nTGCFSGI nTGCFSGI nTGCFSGI nTGCFSGI nTGCFSGI nVcdIYAGie	kqiidlgng. savveng. rgjktneng. skryvnteg. ee.qeesed. rgtkev.ng. ke.degdg. ke.degdg. kgregsgq. kgifetetg. te.dngsgy. hqvvrgvve. rgms. ke.ddgsg rgms. ke.ddgsg. te.dngsgy. hqvvrgvve. rgms. ke.ddgsg. ke.ddgsg. hqvvrgvve. rgms. ke.ddgsg. hqvvrgvve. rgms. ke.ddgsg. hqvvrgvve. rgms. ke.ddgsg. hqvvrgvve. rgms.	ivftpkdgss lvykpsdptt
<pre>(A. tumefaciens ) (B. caldotenax ) (B. napus ) (B. napus ) (B. subtilis ) (C. maltosa ) (C. pasteurianum) (C. utilis ) (C. utilis ) (K. lactis ) (K. marxianus ) (L. interrogan ) (S. pombe ) (S. tuberosum ) (T. aquaticus ) (T. thermophilus) (S. cerevisiae ) (S. cerevisiae )</pre>	80 .gvpyehRPE .dnpphlRPE .hnpaslRPE .gtgalRPE .gtgalRPE .gtgaRPE .gtgavRPE .gtgsvRPE .tlppekqPE .tlppekqPE .tlppekqPE .lgrkiRPE pdgrtdvRPE .glprkiRPE .glprkiRPE eaRvk igk.gh adqtgh dqtgh vg.ygE vg.gi	aG.LLLIRKD KG.LLJIRKE KG.LLJIRKE tG.LLJIRKE tG.LLSIRKQ qG.LLKIRKE ra.LLGLRGA qG.LLKIRKE qG.LLKIRKE rGaLLDLRKH tG.LLALRAG qG.LLKLRKS rSINLLLRKE sG.LLALRKS efnLhKMWKS efnLhKMWKS efnLhKMWKS	100 L. eLFANLRP M. gLFANLRP L. ALYANLRP L. ALYANLRP L. ALYANIRP L. GLYANLRP L. GLYANLRP L. GLYANLRP L. GLYANLRP F. GLFANLRP J. GLFANLRP G. GLFANLRP G. GLFANLRP G. GLFANLRP G. GLFANLRP G. GLFANLRP J. GLFANLRP L. GLFANLRP J. GLFANLRP L. GLFANVRP L. GLYANVRP L. GLYANVRP L. GLYANVRP L. GLYANVRP L. GLYANVRP	aiCY.paLaa vvCY.dsLvs vkaY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle akvY.nvLks cnFasdsLld cnFasdsLld cnFasdsLld cnFasdsLld cnFasksLvk cysLpgyktr akvF.pgLer cqlLspkLad akvF.pgLer gtvFrepii. gtvFrepii. aksiegfktt fksLkgvktr vreFpnvpts	120 aSsLKpElV. rSPLKpDlVq aSPLKrEve aStLKrEva. rSPLKkEyId lSPLrpEvV. aSPLKnEiId lSPIKAEVV. lSPLKpEya. aSPvrsDiIg aSsLKrEvV. ySPLKpEiV. yd lSPLKeE.Ia lSPirn.V. lSPLKeE.Ia ckniprlvpg ipriprlvpr ye yagr	eGlDillVRE g.vDFV1VRE eGvDLmVVRE n.vDLV1VRE kGtnL1VVRE kGtDFVVVRE kGtDFVVVRE nGlDillRE eGvDFVVVRE eGvDFVVVRE eGvDFVVRE eGvDFVVRE eGtDF1VRE rGvDv11VRE wtkpitIgRh wckpitIgRh nv.DLV1RE di.DLVVRE eltDMVIFRE	140 LTGGVYFGEp LTGGIYFGTp LTGGIYFGTp LTGGIYFGTp LTGGIYFGDr LVGGIYFGDr LVGGIYFGEr LVGGIYFGET LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP ahGdqYkatd ahGdqYkatd ahGdqYkatd angGeYsGie ntgGFsGle	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ke.degdg. ke.ddgsg. ke.degdg. kgregsgq. kgifetetg. te.dngsgy. hqvvrgvve. rgms. rgms. fvvdragtfk tlipgpgsle hivcpgvvq. hesvpgvve. hmqtpsvaq.	ivftpkdgss lvykpsdptt vikflreemg
<pre>(A. tumefaciens ) (B. caldotenax ) (B. napus ) (B. subtilis ) (C. maltosa ) (C. pasteurianum) (C. utilis ) (C. utilis ) (K. lactis ) (K. marxianus ) (L. interrogan ) (S. platensis ) (S. pombe ) (S. tuberosum ) (T. aquaticus ) (Y. lipolytica ) (T. thermophilus)</pre>	80 .gvpyehRPE .dnpph1RPE .hnpas1RPE .gtgalRPE .gtgalRPE .gtgaVRPE .gtgsVRPE .gtgsVRPE .lppekqPE .lppekqPE .lpreRPE .lgrkiRPE pdgrtdvRPE .glprkiRPE eaRvk eaRvk igk.gh eaRvk eaRvk eaRvg eaRvg eaRvg 	aG.LLrLRKD kG.LLaiRKq kG.LLgLRKE tG.LLgLRKE tG.LLgLRKG qG.LLkiRKE ra.LLgLRga qG.LLkiRKE qG.LLkiRKE rGaLLpLRKA tG.LLaLRAg qG.LLkLRKS rSlnLtLRKE sG.LLaLRKS qG.LLkLRKS tG.LLsLRKS efkLkkMWKS efnLhkMWKS efnLhkMWKS rSlnLLRKt gSlnvaLRKq ksanvtLRK1	100 L.eLFANLRP M.gLFANLRP L.KVFANLRP L.KVFANLRP L.GLYANLRP L.GLYANLRP L.GLYANLRP L.GLYANLRP L.GLYANLRP L.GLFANTAT M.GVWANLRP L.GLFANTAT M.GVWANLRP L.GLFANTAT G.GLFANLRP G.GLFANLRP D.GLFANLRP L.GLFANLRP L.GLFANLRP L.GLFANLRP L.GLFANLRP L.GLFANLRP L.GLFANLRP L.GLFANVRP L.GLYANVRD	aiCY.paLaa vvCY.dsLvs vkCY.dsLvs vkCY.atLln atvL.pqLvd cnFasdsLle akvY.nvLks cnFasdsLld cnFasdsLld cnFasdsLld cnFasdsLld cnFasdsLld cnFasksLvk cysLpgyktr cqLLspkLad akvF.pgLer gtvFrepii. gtvFrepii. gtvFrepii. dxSiegfktt fksLkgvktr vreFpnvptp vrYYqgtpsp	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aStLKrEva. rSPLKkEyId lSPLrpEvV. aSPLKnEiId lSPLKpEya. lSPLKpEya. aSPVrSDIIg aSsLKrEvV. ySPLKpEiV. yd lSPLKeE.Ia lSPirn.V. lSPLKeE.Ia lSPirn.V. lSPLKeE.ia ripriprlvpr ye yagr	eGlDillVRE g.vDFVIVRE n.vDLVIVRE eGvDLMVVRE kGtDLVVRE kGtDFVVVRE kGtDFVVVRE kGtDFVVVRE cGvDFVVVRE dV.nLITIRE eGvDiMVRE eGtDFIIVRE rGvDvlIVRE rGvDvlIVRE dv.nLITIRE eGtDFIIVRE rGvDvlIVRE gi.DLVVRE eltDMVIFRE	140 LTGGVYFGED LTGGIYFGTD LTGGIYFGTD LTGGIYFGDT LGGIYFGDT LGGIYFGDT LGGIYFGDT LGGIYFGET LGGIYFGET LTGGIYFGET LTGGIYFGED L	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ee.qeesed. rgtkev.ng. ke.ddggg. ke.ddggg. kgifetetg. te.dngsgy. hqvvrgvve. nqms. ke.ddgsg. rgms. ke.ddgsg. rgms. fvvdragtfk tlipgpgsle hivcpgvve. hmqtpsvaq. wkadsadaek	ivftpkdgss lvykpsdptt vikflreemg
<pre>{A. tumefaciens } {B. caldotenax } {B. coagulans } {B. napus } {B. subtilis } {C. maltosa } {C. pasteurianum} {C. utilis } {K. lactis } {K. marxianus } {L. interrogan } {S. pombe } {S. tuberosum } {T. aquaticus } {Y. lipolytica } {T. thermophilus} {S. cerevisiae } }</pre>	80 .gvpyehRPE .dnpph1RPE .hnpas1RPE .knekh1kPE .gtgalRPE .gtgaVRPE .gtgsVRPE .lppekqPE .lprpeRPE .tnpncRPE .glprkiRPE pdgrtdvRPE .glprkiRPE eaRvk eaRvk eaRvk 	aG.LLrLRKD kG.LLaiRKq kG.LLgLRKE tG.LLgLRKE tG.LLsiRKE ra.LLgLRga qG.LLkiRKE rG.LLkiRKE rGaLLpLRKh tG.LLaLRag qG.LLkLRKS rSINLtLRKE sG.LLALRKS rSINLtLRKS rSINLtLRKS rSINLtLRKS rSINLLRKK rSINLLRKK rSINLLRKT rSINVALRKQ kSanVtLRK1	100 L.eLFANLRP M.gLFANLRP L.dLYANLRP L.kvFANLRP L.nLYANLRP L.gLYANLRP L.gLYANLRP L.gLYANLRP L.gLYANLRP J.gLFANLRP J.gLFANTAT M.gvWANLRP Q.dLFANLRP Q.dLFANLRP Q.dLFANLRP D.gLFANVRP L.nLYANLRP G.GLFANLRP L.GLFANLRP L.dLYANLRP L.dLYANLRP L.dLYANVRP L.dLYANVRP	aiCY.paLaa vvCY.dsLvs vkAY.atLln atvL.pqLvd cnFasdsLle akvY.nvLks cnFasdsLld cnFasdsLld aiiY.peLkn llFc.phvld cnFasksLvk cysLpgyktr akvF.pgLer cqlLspkLad akvF.pgLer gtvFrepii. gtvFrepii. gtvFrepii. aksiegfktt fksLkgvktr vreFpnvptp vrYYggtpsp	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aSrLKrEve aSPLKkEyId lSPLrpEvV. aSPLKpEya. lSPLKpEya. aSPVrsDiIg aSsLKrEvV. ySPLKpEiV. ySPLKpEIV. lSPLKEE.Ia lSPirn.V. lSPLKEE.Ia ckniprlvpg ipriprlvpr ye yagr vkhp -SPLK-E-V-	eGlDilIVRE g.vDFVIVRE n.vDFVIVRE eGvDLMVVRE kGtDIIVRE kGtDFVVVRE kGtDFVVVRE kGtDFVVVRE kGtDFVVVRE cGvDinVVRE eGvDFCVVRE rGvDvlIVRE rGvDvlIVRE rGvDvlIVRE rGvDvlIVRE gi.DLVVRE eltDMVIFRE -G-D-VIVRE	140 LTGGVYFGEp LTGGIYFGTP LTGGIYFGTP LTGGIYFGTP LTGGIYFGDT LVGGIYFGDT LVGGIYFGET LVGGIYFGET LTGGIYFGET LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP LVGGIYFGEP ahGdqYkatd ahGdqYAG ahGdqYAG ahGdqYAG ahGdqYAG ahGdqYAG ahGdqYA	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ee.qeesed. rgtkev.ng. ke.ddggg. ke.degdg. kgifetetg. te.dngsgy. hqvvrgvve. rgms. ke.ddgsg. rgms. ke.ddgsg. rgms. fvvdragtfk tlipgpgsle hivcpgvvq. hmqtpsvaq. wkadsadaek	ivftpkdgss lvykpsdptt 
<pre>{A. tumefaciens } {B. caldotenax } {B. coagulans } {B. napus } {B. subtilis } {C. maltosa } {C. pasteurianum} {C. utilis } {K. lactis } {K. marxianus } {L. interrogan } {S. pombe } {S. tuberosum } {T. aquaticus } {T. thermophilus} {S. cerevisiae } {S. cere</pre>	80 .gvpyehRPE .dnpphlRPE .hnpaslRPE .knekhlkPE .gtgatRPE .gtgatRPE .gtgatRPE .gtgstRPE .lppekqPE .lppekqPE .lpreRPE .tnpncRPE .glprkiRPE .glprkiRPE eaRvE eaRvE eaRvE 	aG.LLrLRKD kG.LLgLRKE tG.LLgLRKE tG.LLgLRKE tG.LLgLRKG qG.LLkiRKE rg.LLkiRKE rg.LLkiRKE rGaLLpLRKh tG.LLkLRKS rsinLtLRKE sG.LLkLRKS tG.LLkLRKS tG.LLkLRKS rsinLtLRKE efkLkKMWKS rsinLtLRKE gsinvaLRKQ ksanvtLRK1 rsinvaLRQE -G-LL-LRKE	100 L. eLFANLRP M. GLFANLRP L. dLYANLRP L. dLFANLRP L. GLYANLRP L. GLYANLRP L. GLYANLRP L. GLYANLRP L. GLYANLRP G. GLFANLRP G. dLFANLRP G. dLFANLRP G. dLFANLRP G. dLFANLRP G. dLFANLRP G. dLFANLRP J. GLFANLRP J. GLFANLRP J. GLFANVRP L. GLYANVAN F. eTYANVRP L. dLYICLRP	aiCY.paLaa vvCY.dsLvs vkAY.atLln atvL.pqLvd vkvF.esLsd cnFasdsLle akvY.nvLks cnFasdsLld aiiY.peLkn llFc.phvld cnFasdsLld aiiY.peLkn cysLpgyktr akvF.pgLer cqLlspkLad akvF.pgLer gtvFrepii. gtvFrepii. gtvFrepiv. aksiegfktt fksLkgyktr vreFpnvptp vrYYqgtpsp	120 aSsLKpElV. rSPLKpDlVq aSPLKrErVe aSrLKrEve aSpLKnEiId lSPLrpEvV. aSPLKpEya. ISPLKpEya. ISPLKpEya. aSPVrsDiIg aSsLKrEvV. ySPLKpEiV. ySPLKpEIV. iSPLKeE.Ia lSPirn.V. lSPLKeE.Ia ckniprlvpg ipriprlvpr ye ip	eGlDilIVRE g.vDFVIVRE n.vDFVIVRE eGVDLMVVRE kGtDIIVRE kGtDFVVVRE kGtDFVVVRE kGtDFVVVRE cGVDIVVRE dV.nLIIRE cGVDFCVVRE dV.nLITRE cGVDVIVRE cGVDVIVRE cGVDVIVRE dI.DLIVRE gi.DLVVRE eltDMVIFRE	140 LTGGVYFGEp LTGGIYFGUP LTGGIYFGUP LTGGIYFGUP LTGGIYFGD LUGGIYFGD LUGGIYFGET LUGGIYFGET LUGGIYFGET LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP LTGGIYFGEP ahGdqYkatd ahGdqYkatd ahGdqYkatd mTeGeYSGIe nvedIYaGie nsedIYaGie	kqiidlgng. savveng. serrgpg. rgiktneng. skryvnteg. ee.qeesed. rgtkev.ng. ke.ddggg. ke.degdg. kgifetetg. te.dngsgy. hqvvrgvve. rgms. ke.ddgsg. rgms. ke.ddgsg. rgms. ke.ddgsg. tuchgggsle hivcpgvvq. hesvpgvve. hmqtpsvaq. wkadsadaek	ivftpkdgss lvykpsdptt vikflreemg

			100	,		180		200		
<pre>{A. tumefacie</pre>	ns }	q	krgiDTqiYd	tfEIERIAsv	AFeLArs	RdnrVCSmEK	rNVMk.SgvL	WngVVtEtha	akYk	
<pre>{B. caldotena</pre>	x j	e	ekAvDT11Yk	keEIERIvRM	AFeLArg	RrkkVtSVD <b>K</b>	ANVLs.SSRL	WReVaeEV.a	nEFP	
(B. coagulans)	)	e	nevvDTlaYt	reEIERIiek	AFqLAqi	RrkklaSVD <b>K</b>	ANVLe.SSRM	WReIaeEt.a	kkYP	
<pre>{B. napus</pre>	)	e	evgynTEvYa	ahEIDRIARv	AFetArk	RrgklCSVD <b>K</b>	ANVLd.aSiL	WRrrVtal.a	aEYP	
{B. subtilis	}	e	qeAvDTlfYk	rtEIERViRe	gFkMAat	RkgkVtSVD <b>K</b>	ANVLe.SSRL	WReVaeDV.a	qEFP	
{C. maltosa	}	k	qtAwDTEkYt	vdEVtRItRM	A.afmALqhn	pplpIWS1DK	ANVLa.SSRL	WRrtVdkVis	eEFP	
{C. pasteuria	num}	<b>v</b>	etAfDTEkYn	vdEVkRIAhs	AFkaAmk	RrkkVtSVD <b>K</b>	ANVLd.aSRL	WRKtVnEV.s	kEYP	
{C. utilis	}	<i></i>	.vAsDTEtYS	VDEVERIARM	A.afLALqhn	pplpVWS1DK	ANVLa.SSRL	WRKtVtrVlk	dEFP	
{K. lactis	}		.vAwDsEkYS	VpEVqRItRM	A.afLALqqn	pplpIWS1DK	ANVLa.SSRL	WRKtVeEtik	tEFP	
<pre>{K. marxianus</pre>	}	<i>.</i>	.vAwDsEkYS	vpEVqRItRM	A.afLALqhn	pplpIWS1DK	ANVLa.SSRL	WRKtVeEtik	nEFP	
{L. interroga	n }	e	efAyDTmkYS	rrEIERIAkv	AFqaArk	RnnkVtSID <b>K</b>	ANVLt.tSvF	WkeVViElhk	kEFs	
(S. platensis	}	k	negsnTmaYg	esEIDRIgRv	gFetAkk	RqgrlCSVD <b>K</b>	ANVLd.vSqL	WRdrImal.a	aDYP	
(S. pombe	}	<i>.</i>	AmDTwpYS	leEVsRIARL	A.awLAetsn	ppapVtl1D <b>K</b>	ANVLa.tSRL	WRKtVakIfk	eEYP	
(S. tuberosum	}		slkiit	rqaslRVAey	A.fhYAkthg	Re.rVsaIh <b>R</b>	ANIMqktdgL	FlKccrEVa.	ekYP	
<pre>{T. aquaticus</pre>	}		.vAsDTEtYS	vpEVqRItRM	A.afmALqhn	pplpIWSlDK	ANVLa.SSRL	WRKVVtEtie	kEFP	
<pre>{Y. lipolytic</pre>	a }	e	aeAwnTErYS	kpEVERVAkv	AFeaArk	RrrhltSVDK	ANVLe.vgeF	WRKtVeEV.h	kgYP	
(T. thermophi	lus}	e	aeAwnTErYS	kpEVERVARv	AFeaArk	RrkhVvSVD <b>K</b>	ANVLe.vgeF	WRKtVeEV.g	rgYP	
(pig mitochon		aka wevyn	fpagayamam	vntdEsIsoF	AbscFavAia	kkwolYmst <b>K</b>	ntTLkavdor	FkdIfgEIfe	khYktdfdk.	
(T. thermophi	lusi	amort 1 kvvd	vkasavamam	vntdEsTegF	AhssFkLAid	kklnlFlstK	ntILkkydar	FkdIfgEVve	agYkskfeg.	
(S. ceresesia	e )	adbderuila	siklit	rdasERViRv	A feVArAig	Rn.rVivVhK	stIorladg	FvnVakEls	kEYP.	
(S. cerevisia	e )		slkvmt	roktERIARF	A. fdFAkkyn	Rk.sVtaVhK	ANIMkladaL	FRnIItEIaa	kEYP	
{S. cerevisia	e )		tlklis	wkgsEkIvRF	A.feLArAeq	Rk.kVhcatK	sNIMklaeq.	.pKrafEqva	qEYP	
(E. C	<b>oli</b> )	vkkirfpe	hcgigikpCS	eegtkRlvRa	A.ieYAiAnd	Rd.sVtlVhK	gNIMkftega	Fkdwgyqlar	eEFggelidg	gpwlkvknpn
Conse			A-DTE-YS	EVERIARM	AFALA	RVWSVDK	ANVLSSRL	WRKVV-EV	-EYP	

Fig. 2. Caption appears on facing page.

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(E. coli)

Consensus

	220		240		20	50	280		
<pre>{A. tumefaciens }</pre>	DVaLeHm	LaDaggMQLV	rkPkQFDV	IVTdNLFGDm	LS <b>D</b> v.Aamlt	GSLGMLPSAS	Lga.pdaktg	krkaMYEPVH	GSAPDIAGKs
(B. caldotenax }	DVtLeHm	LVD.mrMQLI	raPkQFDV	<b>IVTeNMFGDI</b>	LSDE.ASmls	GSLGMLPSAS	Lsa.sgp	slyepvh	GSAPDIAGmn
(B. coagulans }	DVeLsHm	LVDstsMQLI	anPgQFDV	<b>IVTeNMFGDI</b>	LSDE.ASvIt	GSLGMLPSAS	Lrs.drf	GMYEPVH	GSAPDIAGqg
(B. napus )	DVeLsHm	<b>yVDnAAMQ</b> LV	rdPkQFDt	IVTnNiFG <b>D</b> I	LSDE.ASmIt	GSIGMLPSAS	Lsd.sgp	GLFEPIH	GSAPDIAGqd
(B. subtilis )	DVkLeHm	LVDnAAMQLI	yaPnQFDV	<b>VVTeNMFGDI</b>	LSDE.ASmlt	GSLGMLPSAS	Lss.sgl	hLFEPVH	GSAPDIAGKg
{C. maltosa }	tlsvqHq	LIDSAchdFn	skPnqI	ewynyhF*					
<pre>{C. pasteurianum}</pre>	EVeLsHl	yVDntAMQLV	rkPsQFDV	IlTnNiFG <b>D</b> I	LSDE.ASmIt	GSiGMLaSsS	ire.dsf	GMYEPIH	GSAPDIAGld
{C. utilis }	qltvqHq	LIDSAAMiLI	KyPtQL.ngI	VITsNMFG <b>D</b> I	iS <b>D</b> E.ASvIp	GSLGLLPSAS	Laslpdsn	kafGLYEPcH	GSAPD1.pan
<pre>{K. lactis }</pre>	qltvqHq	LIDSAAMiLV	KsPtkL.ngV	VITnNMFGDI	iS <b>D</b> E.ASvIp	GSLGLLPSAS	Laslpdtn	kafGLYEPcH	GSAPD1.pan
{K. marxianus }	qltvqHq	LIDSAAMiLV	KsPtkL.ngI	VITnNMFG <b>D</b> I	iS <b>D</b> E.ASvIp	GSLGLLPSAS	Laslpdtn	kafGLYEPcH	GSAPD1.pan
<pre>{L. interrogan }</pre>	DVqLnHl	yV <b>D</b> nAAMQLI	vnPkQFDV	VlceNMFG <b>D</b> I	LSDE.ASiIt	GSIGMLPSAS	Lse.sgf	GLYEPsg	GSAPDIAGKg
{S platensis }	EVeLsHl	yV <b>D</b> nAAMQLV	rwPkQFDt	IVTgNLFG <b>D</b> I	LS <b>D</b> a.Aamlt	GSIGMLPSAS	Lga.sgp	GvFEPVH	GSAPDIAGqd
{S. pombe }	hltLkng	LI <b>D</b> SAAM1LV	KsPrtL.ngV	V1TdNLFG <b>D</b> I	iS <b>D</b> E.ASvIp	GSLGLLPSAS	Lsgvvgksee	kvhcLvEPIH	GSAPDIAGKg
{S. tuberosum }	EIkyeev	vI <b>D</b> nccMmLV	KnPalFDV	1VmpNLYG <b>D</b> I	iS <b>D</b> lcAgl.i	GgLGLtPScn	ige	ggiaLaEaVH	GSAPDIAGKn
{T. aquaticus }	DVaLdHq	yV <b>D</b> amAMhLV	KnParFDV	VVTgNiFG <b>D</b> I	LS <b>D</b> 1.ASvlp	GSLGLLPSAS	L.g.rgt	pvFEPVH	GSAPDIAGKg
<pre>{Y. lipolytica }</pre>	qleLnHq	LI <b>D</b> SAAMiLI	KqPskm.ngI	IITtNMFG <b>DI</b>	iS <b>D</b> E.ASvIp	GSLGLLPSAS	Laslpdtn	eafGLYEPcH	GSAPD1.GKq
{T. thermophilus}	DVaLeHq	yV <b>D</b> amAMhLV	rsParFDV	VVTgNiFG <b>D</b> I	LSD1.ASvlp	GSLGLLPSAS	L.g.rgt	pvFEPVH	GSAPDIAGKg
<pre>{pig mitochond. }</pre>	ykIwyeHr	LIDdmvaQvl	KssggFV	wackNydGDV	qS <b>D</b> ilAqgf.	GSLGLMtSvl	vcpdgktiea	eaahgtvtrH	yrehqkgrpt
(T. thermophilus)	lgIhyeHr	LI <b>D</b> dmvaQMI	KskggFI	malkNydGDV	qSDivAqgf.	GSLGLMtSil	vtpdgktfes	eaahgtvtrH	yrkygkgeet
(S. cerevisiae )	DltLete	LIDnsvLkvV	tnPsaYtdaV	sVcpNLYGDI	LSDlnsglsa	GSLGLtPSAn	igh	.kisiFEaVH	GSAPDIAGqd
(S. cerevisiae )	DIdvssi	iV <b>D</b> nAsMQaV	akPhQFDV	lVTpsMYGtI	LgnigAal.i	GgpGLvagAn	Fgr	dyavFepgsr	hvglDIkGqn
(S. cerevisiae )	DIeavHi	iV <b>D</b> nAAhQLV	KrPeQFEV	IVTtNMnG <b>D</b> I	LS <b>D</b> ltsgl.i	GgLGFaPSAn	ign	e.vaiFEaVH	GSAPkyAGKn

tgkEIvikdv iaDaflqQil lrPaeY..DV IacmNLnGDy iSDalAa.qv GgiGiaPgAn ig.....d e.caLFEatH GtAPkyAGqd

			300	0		320			340	
{A.	<pre>tumefaciens }</pre>	iANPIAmIa.	.SfAMcLRYS	FnMvDE	AtklEaAIan	VLdk	GirTaDimad	gcrqvg	TsDMgDaVla	eFkalsa*
{B.	caldotenax )	KANPIAaIL.	. SAAMMLRLS	FgLtaE	A.ggrarVwq	aLal	Gsgsrlgq.r	rphls	TnEMvEeIka	avldytaiag
{B.	coagulans }	KANPlgtVL.	. SAALMLRYS	FgLekE	AaaIEkAVdd	VLqd	GycTgDlqva	ngkvvs	TiELtDrlie	kLnnsaagpr
{B.	napus }	KANPLATIL.	.SAAMLLkYg	Lgeeka	AkrIEdAVlg	aLnk	GfrTgDiysa	gtklvg	ckEMgEeVlk	svdshvqasv
{B.	subtilis )	mANPfAaIL.	.SAAMLLRtS	FgLeEE	AkaVEdAVnK	VLas	GkrTrDla.r	seefss	TqaitEeVka	aimsentisn
{C.	<pre>maltosa }</pre>									
{C.	pasteurianum}	iANPlAqIL.	.SAAMLMeYS	LdMsEa	ArdVEaAVeK	VLne	GyrTaDiyie	gtkkvg	TsEMgnlVle	rL*
{C.	utilis }	KVNPIAtIL.	.SAAMMLkLS	LdLyEE	gvaVEtAVkq	VLda	GirTgDlkgt	nsttev	gdavaEaVkk	iLa*
<b>{K</b> .	lactis }	KvNPIAtIL.	.SAAMMLkLS	LdLvEE	gralEeAVrn	VLda	GvrTgDlggs	nsttev	gdaiakaVke	iLa*
(K.	marxianus }	KvNPIAtIL.	.SAAMMLkLS	LdLvEE	graVEeAVrK	VLds	GirTgDlggs	nsttev	gdavakaVke	iLa*
{L.	interrogan }	VANPIAqVL.	. SAALMLRYS	FsMeEE	AnkIEtAVrK	tias	GkrTrDiaev	gstivg	TkEigqlIes	fL*
(S.	<pre>platensis }</pre>	KANPlAqVL.	. SAAMMLRYg	Ldepaa	sdpVEkAV1K	VLdw	GypTgDimse	gmkavg	crkwgicy	
{S.	pombe }	ivNPVgtIL.	. SAsLLLRYg	LnapkE	AeaIEaAVrK	VLddtsiggr	GlyTrDlgge	astadi	TkavvEelek	iL*
{S.	tuberosum }	lANPtAllL.	.SsvsMLRh.	LeLhDk	AdrIqdAIlK	tiaggkvpnw	rpwrh	cyn	n*	
{T.	aquaticus }	iANPtAaIL.	.SAAMMLeha	FgLvEl	ArrVEaAVaK	aLre	tpppdlggsa	g	TqaFtEeVlr	hL*
{Y.	lipolitica }	KVNPIAtIL.	.SAAMMLkFS	LnMkpa	gdaVEaAVke	svea	GitTaDiggs	sstsev	gdlLptrsrs	csrrsksflr
{ <b>T</b> .	thermophilus}	iANPtAaIL.	.SAAMMLeha	FgLvEl	ArkVEdAVaK	aLle	tpppdlggsa	g	TeaFtatVlr	hLa
{pig	g mitochond. }	stNPIAsIFa	wtraLehRak	LdgngdLirf	AgtlEkvcve	tv.esgamtk	dlagcihgls	nvklnehfln	TsDFlDtIks	nLdralgrg.
(T.	thermophilus}	stNsIAsIFa	wSraLLkRae	LdntpaLckf	AnilEsAtln	tvaadaimtk	dlalac	gnnersavvt	TeEFlDaVek	rLokeiksie
{S.	cerevisiae }	KANPtAllL.	.SsvMMLnh.	MgLtnh	AdgIgnAVls	tiasgpenrt	Gdlag	tat	TssFtEaVik	rL*
{S.	cerevisiae }	vANPtAmIL.	.SstLMLnh.	LqLnEv	AtrIskAVhe	tiaegk.htt	rdiga	sss	TtDFtneIin	kLstm
{S.	cerevisiae )	viNPtAvlL.	. SAVMMLRY.	Leefat	AdlIEnAlly	tLeegrvltg	dvvgydr	gak	TtEvtEalig	nLaktprkta
-	{E. coli}	KvNPasiIL.	.SAeMMLRh.	MgwtEa	Adlīvkamea	ai.naktvtv	dferlmd	gakllk	csEFgDalie	nM*
				-						

Consensus KANPIA-IL- -SAAMMLRYS L----L-EE A--IE-AV-K VL------ G--T-D---- ----- T-EF-E-V-- -L------

Fig. 2. Alignment of amino acid sequences of isopropylmalate dehydrogenases (IPMDH) and isocitrate dehydrogenases (ICDH). All sequences are retrieved from GenBank. Capital letters indicate that the residues are conserved in 10 of the 24 total sequences. The consensus sequence is also listed at the bottom of the aligned individual sequences. Sources of the IPMDH sequences and their corresponding GenBank codes are: Amyloliquefaciens tumefaciens, atumadh15; Bacillus caldotenax, bc3imd; Bacillus coagulans, bacipmd; Bacillus napus, bnipmdh; Bacillus subtilis, bsleuc; Clostridium maltosa, cmlebbid; Clostridium pasteurianum, cloleuily; Clostridium utilis, ysaimdh; Klebsiella lactis, klklleu2g; Klebsiella marxianus, ekleu2g; Leptospira interrogans, lepleub; Staphylococcus platensis, spu3id; Schizosaccharomyces pombe, yspleu1a; Staphylococcus tuberosum, tbbisodeh; Thermus aquaticus, d10700; Thermus thermophilus, tthleub; Yersinia lipolytica, ysjleu2b. Sources of the ICDH sequences and their corresponding GenBank codes are: pig mitochond., pigmtnadp; T. thermophilus, tthisocitd; Saccharomyces cerevisiae (NADPH-specific), yscidp1; S. cerevisiae, yscidh2a; S. cerevisiae (mitochond.), yscisodh; E. coli, ecoicd.

241 and 245) and ICDH (D283', 307 and 311) also align with each other. The active site residues Y160 and K230', which primarily interact with the C6 carboxyl oxygens of isocitrate in ICDH, superimpose with the corresponding Y139 and K185' in IPMDH.

Structural alignment of the two proteins also reveals their distinctive features. First, the interdomain pocket of IPMDH that contains the active site is significantly larger than that of ICDH (Fig. 3A,B). No large binding-induced conformational changes were observed in the co-crystal structure of ICDH with isocitrate and NADP except for some minor conformational changes

in the active site (Stoddard et al., 1993). If IPMDH has the same substrate and coenzyme binding modes as ICDH, more than half of the binding pocket would be occupied by solvent molecules, whereas the ICDH has tight packing of isocitrate and coenzyme with few solvent molecules. Therefore, binding-induced conformational change such as an interdomain hinge motion is likely. In the liver alcohol dehydrogenase (ADH), for example, the binding of coenzyme NAD induces a conformational change of the enzyme in which the protein appears to gain favorable contacts with the coenzyme and reduces the solvent-accessible sur88





Fig. 3. Structural alignment of T. thermophilus IPMDH and E. coli ICDH. Only the secondary structures around the active sites are shown. Despite good alignments of the  $\beta$ -strands and the  $\alpha$ -helices on the righthand side, several helices of IPMDH on the left-hand side are displaced from those corresponding ones of ICDH. An interdomain hinge motion will align these helices of IPMDH to those corresponding helices of ICDH. The hinge axis is displayed in red and the Mg<sup>2+</sup> ion (red) approximately indicates the center of the active site. A: Side view of the ICDH active site and the aligned IPMDH. The structure of ICDH is shown in yellow and IPMDH in cyan. B: Top view of the active site of ICDH and the aligned IPMDH. The structure of ICDH is in shown yellow and IPMDH in cyan. C: Alignment of the catalytic residues in the active sites of IPMDH (black) and ICDH (white). The IPMDH residues are labeled with single-letter amino acid codes. The corresponding IDH residue are numbered in the parentheses, except S113 and N115, which are displaced from the corresponding T88 and L90, respectively.

face area (Eklund et al., 1984). In the case of IPMDH, a similar hinge motion would also close down the interdomain binding pocket (Fig. 3A) and create favorable contact with the substrate and the coenzyme. This hinge motion also would make better alignment of the helices of the outside domains of the two proteins (Fig. 3A,B).

#### Docking isopropylmalate to IPMDH

To investigate the possible substrate binding modes of IPMDH, isopropylmalate and NAD were docked separately to IPMDH by the automated Monte Carlo docking method of Goodsell and Olson (1990). The "short" and "long" docking schemes produced no significant difference in the docking positions (see Methods). The best docking solutions of isopropylmalate and NAD by the "long" scheme are shown in Figures 4A and 5A, and the calculated energies of the top-ranked docking solutions are listed in the figure legends. The calculated docking energy is, of course, not the absolute binding energy per se, but a relative indication of how favorably a substrate interacts with a protein. The conformations of the bound isocitrate and NADP in ICDH are also shown for comparing the substrate binding modes of the two proteins (Figs. 4B, 5B).

Docking of isopropylmalate with the "long" scheme resulted in 10 different binding positions with calculated energies ranging from -30.6 to -26.8 kcal/mol. The three binding positions with the best calculated energies (-30.6 to -29.5 kcal/mol) are very similar and therefore considered to be one binding mode (Fig. 4A). Because the predominant forces in the docking forcefield were hydrogen bonding and charge-charge interactions, the automated docking resulted in a binding mode that placed the carboxyl and hydroxyl groups of isopropylmalate close to the charged amino acids, i.e., glutamates and arginines. For example, the  $\alpha$ -carboxyl group of isopropylmalate makes close contacts with the side chain atoms of R94, N102, R132, L134, and S261. The  $\beta$ -carboxyl group interacts with residues R104, S259, V272, and the nicotinamide ring of the docked coenzyme NAD. The hydroxyl group forms charge-charge interactions with the R132 and a possible bound  $Mg^{2+}$  (see below).

The coenzyme NAD was first docked to IPMDH in the absence of bound isopropylmalate molecule. The top docking solutions of both "short" and "long" schemes converged to a single binding mode as shown in Figure 5A. The docked NAD took



**Fig. 4.** A: Docked conformation of isopropylmalate (gray) and the surrounding residues of the *T. thermophilus* 3-isopropylmalate dehydrogenase (black). Active site residues are labeled with single-letter amino acid codes. The "long" docking scheme resulted in solutions with calculated energies ranging from -30.6 to -26.8 kcal/mol. The binding mode shown was derived from the top three docking solutions with calculated energies ranging from -30.6 to -29.5 kcal/mol. B: Binding conformation of isocitrate (gray) in the active site of *E. coli* isocitrate dehydrogenase (white).

an extended conformation with the nicotinamide ring slightly folding back toward the diphosphoester group. In this binding mode, the nicotinamide ring is close to several conserved residues in IPMDH, including R104, D245, L254, V272, and the  $\beta$ -carboxyl group of the docked isopropylmalate molecule. The hydroxyl groups of the nicotinamide side sugar ring make contact with the residues L90, R94, and E87. The adenine group of NAD makes contact with L91, R94, K95, R132, L134, Y139, and P140. Most of these residues are conserved in IPMDHs from different species (Fig. 2).

The most important feature of this NAD binding mode is that the nicotinamide ring makes close contact with the  $C_2$  atom of the docked isopropylmalate. This close contact facilitates the hydride transfer reaction between the  $C_2$  atom of isopropylmalate and the  $C_4$  atom of the nicotinamide ring of NAD. Such hydride transfer reactions were proposed as essential steps in the catalytic mechanisms of both IPMDH and ICDH. The rest of the docked NAD also makes favorable contact with isopropylmalate but without any overlapping, even though the two molecules were docked independently. Reproducing these essential interactions between substrate and coenzyme by the independent docking of each molecule supports the docking-derived binding modes of substrate and coenzyme in IPMDH.

In addition to the coenzyme NAD, the catalysis of isopropylmalate by IPMDH needs a cation, either a  $Mg^{2+}$  or a  $Mn^{2+}$ (Imada et al., 1991). The bound  $Mg^{2+}$  stabilizes the charged intermediate during the dehydrogenation reaction. In ICDH, the Mg<sup>2+</sup> coordinates with several aspartate residues and the carboxyl and hydroxyl group of the substrate (isocitrate). Considering the sequence and structural homology between IPMDH and ICDH, we assumed that the cation required in the function of IPMDH played similar role as the  $Mg^{2+}$  ion in the catalysis of ICDH. The binding position of the Mg<sup>2+</sup> was determined by docking to IPMDH-isopropylmalate complex. All docked positions essentially converged to one binding site, which is shown with the docked isopropylmalate and NAD in Figures 4A and 5A. In this binding position, the  $Mg^{2+}$  can form coordinating interactions with the conserved catalytic residues D307, D311, and D283' of IPMDH and the  $\alpha$ -, $\beta$ -carboxyl groups of the docked isopropylmalate. The negatively charged phosphodiester group of the docked NAD molecule may also interact with the Mg<sup>2+</sup> ion (Fig. 5A). Similar coordinating interactions between the bound cation and the enzyme/substrate were also observed in the active site of ICDH (Figs. 4B, 5B).

The binding of isopropylmalate, NAD, and  $Mg^{2+}$  are consistent with the proposed catalytic mechanism of IPMDH. The proposed reaction starts with the base-catalyzed removal of a proton from the hydroxyl group of the isopropylmalate. The base may be one of the nearby aspartates such as D241 or D245. The deprotonated intermediate is stabilized by the bound cation and the surrounding polar residues, e.g., aspartates, arginines, etc. (Fig. 4A). The dehydrogenation induces the transfer of a hydride from the intermediate to the cofactor NAD<sup>+</sup>, and the substrate isopropylmalate is then converted to oxaloisoval-



**Fig. 5.** A: Docked conformation of nicotinamide adenine dinucleotide (NAD, black) and isopropylmalate (gray) in the active site of *T. thermophilus* 3-isopropylmalate dehydrogenase. The "long" docking scheme resulted in solutions with calculated energies ranging from -68.3 to -55.0 kcal/mol. The binding mode shown was derived from the top four docking solutions with calculated energies ranging from -68.3 to -64.1 kcal/mol. B: Binding conformation of nicotinamide adenine dinucleotide phosphate (NADP, white) and isocitrate (gray) in the active site of *E. coli* isocitrate dehydrogenase.

erate. The close contact between the nicotinamide ring nitrogen of the NAD and the C<sub>2</sub> atom of isopropylmalate would facilitate this transfer. In the second step, the  $\beta$ -carboxylate of oxaloisovalerate is lost as CO<sub>2</sub>, and the concomitant protonation of the  $\beta$ -carbon transforms oxaloisovalerate to  $\alpha$ -ketoisocaproate.

Despite these common features, the substrates of IPMDH and ICDH showed key differences in their interactions with the enzymes and coenzymes. Although the nicotinamide rings of both coenzymes make close contact with the C<sub>2</sub> atoms of substrates, in ICDH, the only other contact between NADP and isocitrate involves the nicotinamide ring of NADP and the  $\gamma$ -carboxyl group of isocitrate, and the phosphodiester does not make direct contact with isocitrate. In IPMDH, however, the  $\gamma$ -moiety of the docked isopropylmalate is placed next to the phosphodiester group. The  $\alpha$ -carboxyl group faces the adenine ring of NAD. These differences provide an explanation for the different substrate specificities of IPMDH and ICDH. ICDH shows

no catalytic activity against isopropylmalate because replacing the  $\gamma$ -carboxyl group with a nonpolar isopropyl group would remove a favorable, specific interaction with ring nitrogen of the nicotinamide of NADP, which orients hydride donor (C2) on the substrate to the hydride acceptor  $(C_4)$  on the coenzyme. The docked isopropylmalate and NAD in IPMDH interacts differently. Because the  $\gamma$ -moiety is placed next to the diphosphoester group of NAD, replacing it with a negatively charged carboxyl group (isopropylmalate → isocitrate) would create repulsive interactions between NAD and isopropylmalate. This repulsive interaction will destabilize the tertiary binding complex and especially alter the orientation of hydride donor on the substrate and acceptor on the substrate. Therefore, the rate of the hydride transfer reaction would be decreased. On the other hand, replacing the  $\gamma$ -moiety with other nonpolar groups seems less destructive to the interaction between isopropylmalate and NAD because the packing interaction around the  $\gamma$ -moiety is not very specific. Kinetic studies of both ICDH and IPMDH confirmed that the catalysis is less sensitive to the length of alkyl groups at the  $\gamma$  position (Miyazaki et al., 1993). In addition, the  $\beta$ -carboxyl of IPM may interact favorably with the NAD.

IPMDH and ICDH use different coenzymes NAD and NADP, respectively. In ICDH, the extra phospho group of the bound NADP interacts with several arginines and tyrosines residues (R292', R395, Y345, and Y391). In IPMDH, sequence alignment showed that at least one of the arginines (R395) and the two tyrosines are absent. These lost interactions may explain the distinct binding mode of NAD in IPMDH. On the other hand, the adenine sugar makes close contact with the  $\alpha$ -carboxyl group of the bound isopropylmalate and the active site residue R132 (Fig. 4A, 5A) with the average distances of 6 Å and 5 Å, respectively. Adding an extra phospho group to the adenine ribose will create strong interactions with the substrate and R132 and change the relative binding orientations between the substrate and coenzyme.

The binding of isopropylmalate in IPMDH also differs slightly from that of isocitrate in ICDH. In ICDH, the bound isocitrate interacts directly with several aspartates in the active site, such as D283', D307, and D311. However, the docked isopropylmalate in the active site of IPMDH is displaced from the corresponding aspartates. The distances between the docked isopropylmalate and Y139 and K185' are also significantly longer than between their counterparts in the active site of ICDH (Y160, K230', see Fig. 4). This different binding mode of isopropylmalate seems consistent with the recent observation that mutating Y139 to phenylalanine had little effect on binding of isopropylmalate (Miyazaki & Oshima, 1993). The predominant effect of this Y139  $\rightarrow$  F mutation is on the binding of NAD, which is consistent with the docked position of the coenzyme, where the Y139 is in close contact with the adenine ribose ring (3.3 Å).

#### Conclusions

A docking algorithm and sequence homology between the *T. thermophilus* IPMDH and *E. coli* ICDH have allowed us to identify the binding site of isopropylmalate. The substrate and coenzyme (NAD) binding modes of IPMDH and ICDH have striking similarities and some important differences. The catalytic residues and metal ion bindings are very similar, but the cofactor bindings are different. The difference in the cofactor binding mode, together with the different side chains of the substrates, lead to the differences identified with substrate specificities. In addition, the contrast between the tightly packed ICDH site and the loosely packed IPMDH site suggests a ligand-induced hinge motion in IPMDH during the enzyme action.

## Methods

#### Sequence and structure alignments

The amino acid sequences of ICDH and IPMDH from different species were translated from the DNA or mRNA sequences reported in GenBank. The homologous alignment of these sequences was done with the Needleman–Wunsch algorithm (Needleman & Wunsch, 1970).

The structural alignment of ICDH and IPMDH was done by using a transformation matrix derived from the nonlinear leastsquare fitting of selected residues from both proteins. First, the atoms of these selected residues were fitted to each other to derive the transformation matrix. The transformation matrix was then used to superimpose the two protein structures. The residues selected in ICDH are: R129, R153, Y160, K230', D283', D307, and D311. These residues were selected because they were shown to play important roles in the enzyme function (Dean & Koshland, 1990, 1993). Based on sequence alignment of ICDH and IPMDH, the corresponding residues in IPMDH are: R104, R132, Y139, K185', D217', D241, and D245 (Fig. 2). Coordinates of IPMDH and ICDH were taken from the known crystal structures of the two proteins deposited in the Brookhaven Protein Data Bank (1ipd and 5icd).

#### Monte Carlo docking

To determine the bound conformation of isopropylmalate on IPMDH, we used the automated docking method of Goodsell and Olson (1990). This method has been described previously and only a brief description is given here (Goodsell & Olson, 1990). In the automated docking method, a flexible substrate performs a random walk around the surface of a static protein to search an optimal binding site. At each search step, the substrate was translated and rotated about its center and each of its torsional angles while its interaction energy with the protein was evaluated. The acceptance or rejection of a configuration was based on the interaction energy and a temperature factor. This large configurational space was sampled by the simulated annealing method. The search starts at high temperature, so the interaction energy has little impact on the acceptance or rejection of a configuration. This allows a relatively unrestricted search of the configurational space. As the simulation proceeds, the temperature is lowered and the impact of interaction energy on the acceptance or rejection is increased. The substrate was therefore restricted to only energetically favorable configurations. Multiple runs are carried out to increase the sampling of this configurational space.

The enzyme-substrate interaction energy was evaluated in terms of a molecular mechanic force field. The force field includes electrostatic, hydrogen bonding, and dispersion/repulsion terms and was able to reproduce crystallographic binding modes of several polar substrates (Goodsell & Olson, 1990). We also tested the force field by docking isocitrate to the E. coli ICDH. The best docking solution of isocitrate agrees with known crystallographic structure of ICDH-isocitrate complex. The docking calculation was speeded up by use of a precalculated potential map of a cubic search region. The structure of IPMDH was taken from the 2.2-Å crystal structure of the apo protein (Imada et al. 1991; PDB code: 1ipd). The missing polar hydrogens in the structure were modeled by the H-build routine of Charmm22 (Brooks et al., 1983). The atomic partial charges of the protein assigned according to the Charmm22 extended atom force field. The atomic partial charges of isopropylmalate were determined by fitting the electrostatic potential derived from the semi-empirical quantum mechanical molecular orbital model (PM3).

The search for the binding position of isopropylmalate was confined to the potential active site of IPMDH as identified by structural and sequence homology with ICDH. A "crude" search was first conducted with a 50-Å cubic box of a 0.5-Å grid. A "fine" search was then followed with a 25-Å cubic box of 0.25-Å grid. The torsional angles of each flexible bonds have been kept flexible in the docking process. Several distance constraints were used to prevent overlapping of atoms that are separated by more than three bonds. The substrates were docked by two different docking schemes. In the "short" docking scheme, substrates were docked with 100 independent docking runs, each with a different random seed number. Each run contains 50 cycles of 3,000 steps accepted or rejected, starting at a high temperature ( $k_BT = 100$  kcal/mol) and decreasing by a factor of 0.9 each cycle. In the "long" docking scheme, the substrates were docked with 10 individual docking runs; each run has 50 cycles of 30,000 steps accepted or rejected while the temperature parameters are

kept the same. The top-scored configurations given by both docking schemes were very similar. The results reported in this study were those from the "long" docking scheme (30,000 steps accepted or rejected).

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#### References

- Brooks B, Bruccoleri R, Olafson B, States D, Swaminathan S, Karplus M. 1983. Charmm: A program for macromolecular energy, minimization and molecular dynamics calculations. J Comput Chem 4:187-217.
- Colman R. 1973. A glutamyl residue in the active site of triphosphopyridine nucleotide-dependent isocitrate dehydrogenase of pig heart. J Biol Chem 248:8137-8143.
- Dean A, Koshland DE Jr. 1990. Electrostatic and steric contributions to regulation at the active site of isocitrate dehydrogenase. *Science 249*:1044– 1046.

- Dean A, Koshland DE Jr. 1993. Kinetic mechanism of *Escherichia coli* isocitrate dehydrogenase. *Biochemistry* 32:9302-9309.
- Ehrlich R, Colman R. 1978. Histidine in the nucleotide-binding site of NADP-linked isocitrate dehydrogenase from pig heart. Eur J Biochem 89:575-587.
- Eklund H, Samama J, Jones T. 1984. Crystallographic investigations of nicotinamide adenine dinucleotide binding to horse liver alcohol dehydrogenase. *Biochemistry* 23:5982-5996.
- Goodsell D, Olson A. 1990. Automated docking of substrates to proteins by simulated annealing. *Proteins Struct Funct Genet* 8:195-202.
- Grissom C, Cleland WW. 1985. Use of intermediate partitioning to calculate intrinsic isotope effects for the reaction catalyzed by malic enzyme. *Biochemistry* 12:944-948.
- Hurley J, Dean A, Koshland DE Jr, Stroud R. 1991. Catalytic mechanism of NADP<sup>+</sup>-dependent isocitrate dehydrogenase: Implications from the structures of magnesium-isocitrate and NADP<sup>+</sup> complexes. *Biochemistry* 30:8671-8678.
- Imada K, Sato M, Tanaka N, Katsube Y, Matsuura Y, Oshima T. 1991. Three-dimensional structure of a highly thermostable enzyme, 3-isopropylmalate dehydrogenase of *Thermus thermophilus* at 2.2 Å resolution. J Mol Biol 222:725-738.
- Kornberg H. 1966. The role and control of the glyoxylate cycle in Escherichia coli. Biochem J 99:1-11.
- Miyazaki K, Eguchi H, Yamagishi A, Wakagi T, Oshima T. 1992. Molecular cloning of the isocitrate dehydrogenase gene of an extreme thermophile, *Thermus thermophilus* hb8. *Appl Environ Microbiol* 58:93-98.
- Miyazaki K, Kakinuma K, Terasawa H, Oshima T. 1993. Kinetic analysis on the substrate specificity of 3-isopropylmalate dehydrogenase. FEBS Lett 11:35-36.
- Miyazaki K, Oshima T. 1993. Tyr 139 in *Thermus thermophilus* 3-isopropylmalate dehydrogenase is involved in catalytic function. *FEBS Lett* 11:37-38.
- Needleman S, Wunsch C. 1970. A general method applicable to the search for similarities in the amino acid sequences of two proteins. J Mol Biol 48:443-453.
- Pirrung M, Han H, Nunn D. 1994. Kinetic mechanism and reaction pathway of *Thermus thermophilus* isopropylmalate dehydrogenase. J Org Chem 59:2423–2429.
- Stoddard B, Dean A, Koshland DE Jr. 1993. Structure of isocitrate dehydrogenase with isocitrate, nicotinamide adenine dinucleotide phosphate, and calcium at 2.5-Å resolution: A pseudo-Michaelis ternary complex. *Biochemistry* 32:9310–9316.