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Design and synthesis of new enzymes based on the lactate dehydrogenase framework

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[Plate 1]

SUMMARY

Analysis of the mechanism and structure of lactate dehydrogenases is summarized in a map of the catalytic pathway. Chemical probes, single tryptophan residues inserted at specific sites and a crystal structure reveal slow movements of the protein framework that discriminate between closely related small substrates. Only small and correctly charged substrates allow the protein to engulf the substrate in an internal vacuole that is isolated from solvent protons, in which water is frozen and hydride transfer is rapid. The closed vacuole is very sensitive to the size and charge of the substrate and provides discrimination between small substrates that otherwise have too few functional groups to be distinguished at a solvated protein surface. This model was tested against its ability to successfully predict the design and synthesis of new enzymes such as L-hydroxyisocaproate dehydrogenase and fully active malate dehydrogenase. Solvent friction limits the rate of forming the vacuole and thus the maximum rate of catalysis.

INTRODUCTION

The rational design of new protein-based devices is possible because advanced computer-controlled chemical synthesis makes available DNA fragments of defined sequence, which can be biologically translated into a polypeptide chain of any chosen sequence of amino acids. Most biological properties of a protein depend upon the extended polypeptide chain folding into a unique three-dimensional structure. Synthetic polypeptide sequences do not usually fold fast enough to avoid tangled precipitates or survive attack by cellular degradation systems and thus protein engineers currently making new proteins avoid the kinetic folding problem by only altering small parts of otherwise large, stable and rapidly self-folding protein frameworks. This we call protein redesign as opposed to *de novo* protein design. Redesign requires a correct analysis of the natural protein design, but difficulties arise in distinguishing features important for the accepted properties (as an enzyme, hormone, etc.) from those important for more cryptic, often unsuspected biological functions. One way to avoid such red herrings is to test analytical conclusions against their success in predicting the synthesis of structures with predetermined functions. This paper describes this approach applied to the enzyme NAD$^+$: L-lactate oxidoreductase.

ANALYSIS OF THE NATURAL ENZYME

The relation of function to the lactate dehydrogenase framework is summarized by a map of the catalytic pathway (figure 1). The established chemical mechanism is direct stereospecific transfer of a hydride ion from the C_4-position of the dihydronicotinamide ring to directly reduce a carbonyl (Westheimer 1987). The free protein obligatorily binds each coenzyme before the small substrate at a histidine–aspartate couple of defined protonation state. The diagram shows the rapid (> 1000 s^{-1}) reversible two-electron equilibrium between a protonated histidine–aspartate couple through the keto—hydroxy-acid bond to the nicotinamide ring. This map explains many experimental observations, namely the neutral dihydropyridine ring of NADH binds much tighter than the positively charged ring of NAD$^+$; ternary complexes of NADH and a ketoacid are compulsorily protonated and substrate can force a proton onto the histidine–aspartate couple against a 10^4 unfavourable bulk proton concentration (Holbrook 1973); dihydropyridine adducts formed from NAD$^+$ and sulphite, cyanide or enolpyruvate are stabilized by up to 10^5 compared with the stability free in solution (Parker & Holbrook 1977; Lodola *et al.* 1978); stabilization 'on-enzyme' of NADH and pyruvate by 10^3 over the free solution equilibrium (Holbrook & Gutfreund 1973; Clarke *et al.* 1988). These perturbed 'on-enzyme' equilibria reflect a very hydrophobic environment around the pyridine ring which, in an environment shielded from solvent, selects for neutral as opposed to charged adducts (Parker & Holbrook 1977). The tests included: making the pyridine ring environment more hydrophilic (Ile 250 → Asn) – this equalizes affinity for NAD$^+$ and NADH (Wigley *et al.* 1987*a*). Replacing Asp 168 by Ala or Asn selectively destabilizes the protonated His 195/NADH carbonyl groundstate compared to the neutral His 195/NAD$^+$/carbinol groundstate (Clarke *et al.* 1987*a*) and pre-resonance laser Raman spectra show the carbonyl to then have only 20 % single bond character compared with 40 % single bond in the wild-type ground state (Deng *et al.*

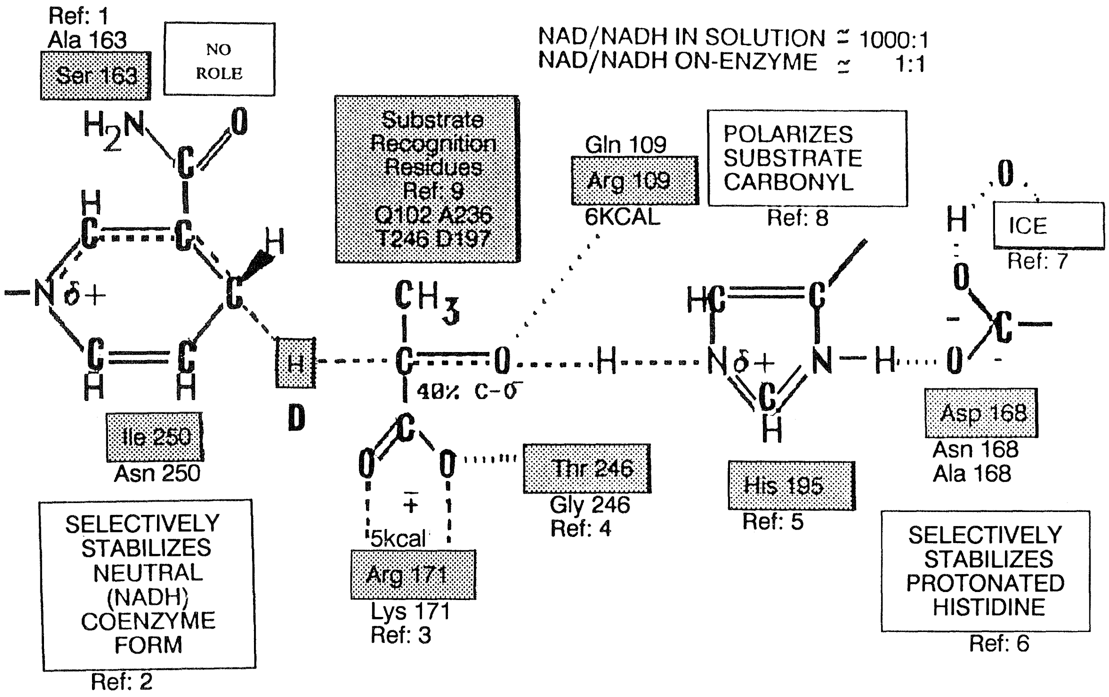

Figure 1. A map showing the roles of amino acids around the extended lactate dehydrogenase catalytic pathway of *B. stearotherophilus* lactate dehydrogenase. Original results are for Ref. 1: Wigley *et al.* 1987*b*. Ref. 2: Wigley *et al.* 1987*a*. Ref. 3: Hart *et al.* 1987*a*, *b*; Wigley *et al.* 1987*c*. Ref. 4: Clarke *et al.* 1987*a*, Bur *et al.* 1989. Ref. 5: Holbrook 1973; Holbrook & Ingram 1973; Lodola *et al.* 1978. Ref. 6: Clarke *et al.* 1988. Ref. 7: this paper. Ref. 8: Clarke *et al.* 1986. Ref. 9: Clarke *et al.* 1987*a*; Wilks *et al.* 1988, 1990.

1990). Arginine 109 (on the underside of the mobile surface loop) hardly stabilizes the stretched carbonyl in the groundstate, but compared with a glutamine-109 mutant, it decreases the transition state barrier by 4–6 kcal mol^{-1} (Clarke *et al.* 1986). Pathway residues are conserved in all LDH sequences but some, such as Ser 163, may be changed (e.g. to Ala 163) without measurable effect. Residues 109 and 102, which are important for catalysis and for substrate recognition, are on the underside of the mobile loop (98–110) and only take up the position shown after the substrate-induced conformation change.

A SUBSTRATE-INDUCED PROTEIN SHAPE CHANGE LIMITS CATALYSIS

To move a substrate into an internal, proton- and solvent-isolated vacuole requires the protein change between at least two shapes: one where the internal pocket is open to the solvent so the ionic substrate can enter and another closed state which has established the catalytic pathway. Kinetic experiments similar to that shown in figure 2*d*, plate 1 for pig M4 LDH show: (i) the maximum rate of conversion of pyruvate to lactate was uninfluenced by changing the hydrogen

DESCRIPTION OF PLATE 1

Figure 2. (*a*–*g*) Crystallographic and kinetic description of the substrate induced conformation change. (*a*) The packing of molecules of pig M_4 lactate dehydrogenase in crystal of the enzyme with NADH and oxamate from which half the oxamate has been washed out. Because of the 26° tilt between adjacent molecules in the crystal lattice, the mobile specificity loop (coloured white) of one of the two subunits in the asymmetric unit (coloured orange in (*a*)) is much closer to the next tetramer than that of the other subunit (red in (*a*)). In the orange subunit the specificity loop (picked out in grey) cannot open because it contacts the next tetramer in the lattice: this active site is still ternary i.e. contains both NADH and oxamate (*f*). In the red, unrestrained subunit the loop has partly opened and the active site contains no oxamate i.e. is a binary structure (*g*). (*b*) The Cα backbones of the binary (yellow) and ternary (deep blue) subunits superimposed. Red dots mark residues conserved in 21 sequences. The coenzyme and oxamate are coloured red. Where the subunits exactly match one grey trace is seen. (*c*) A schematic of a subunit of *B. stearothermophilus* lactate dehydrogenase complex with NADH, oxamate (picked out as a ball stick) and fructose-1,6-bisphosphate and oxamate with the secondary structures labelled. The bold trace is the specificity and catalysis loop (98–110), which opens and closes to release and accept substrate. (*d*) Stopped flow trace of the maximum rate of oxidation of NADH by pig M_4 enzyme and a saturating concentration of pyruvate, pH 7.2, 25 °C. This rate is 320 s^{-1} when NADD ([nicotinamide-4-^{2}H]NADH) is used instead of NADH. (*e*) The binding of saturated concentrations of a substrate–analogue, oxamate, is limited by the same conformation change, this time monitored by the change in tryptophan fluorescence. (*f*) The electron density around the ternary active site. Note the strong density for waters close to Glu 194, Arg 109, Asp 168 and Asn 140, which are absent or much weaker in the (g) electron density map for the binary complex.

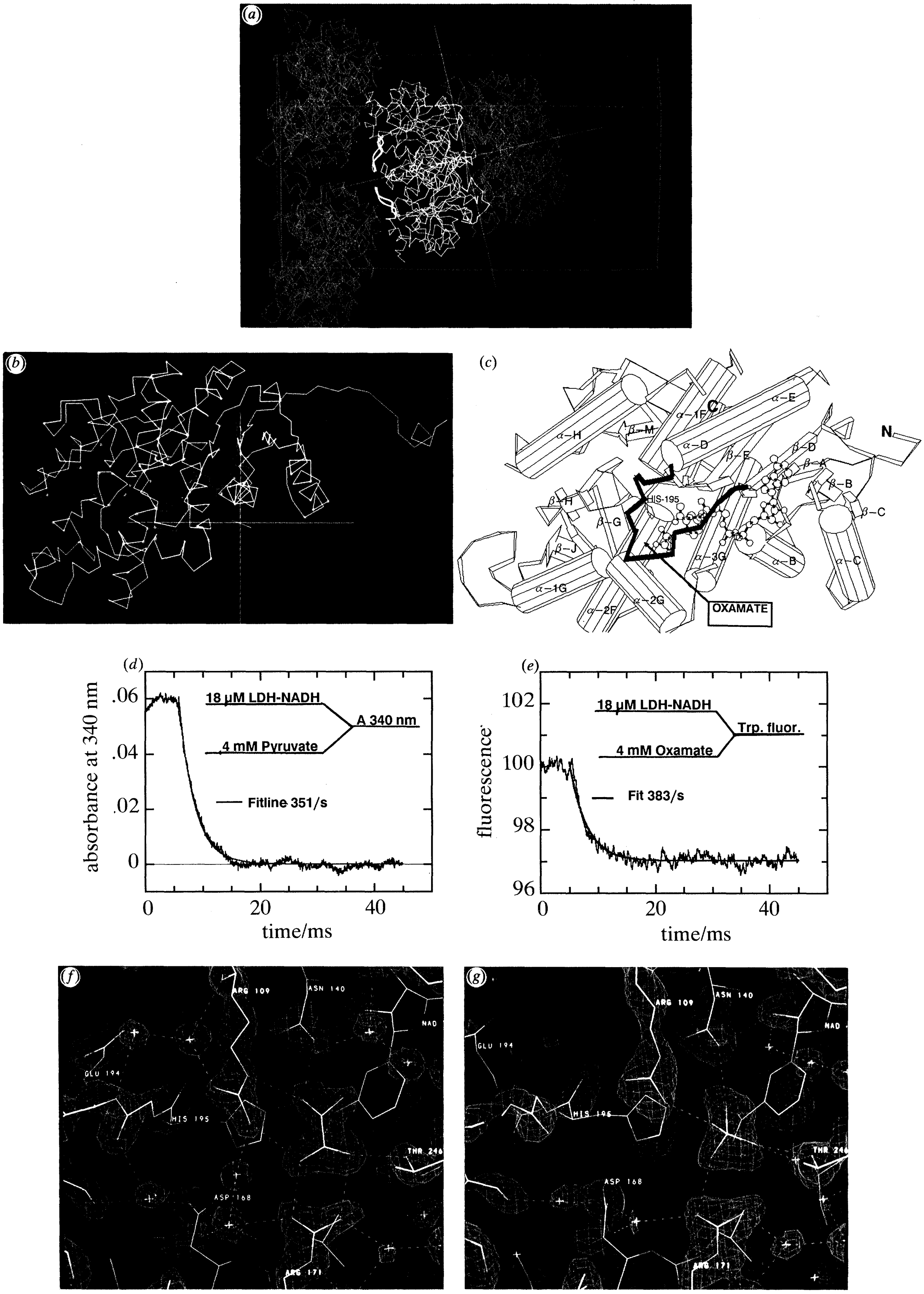

Figure 2. For description see opposite.

which is transferred to deuterium (whereas when C—H bond breaking is the slowest step, deuterium substitution slows rates by 3–7 fold, e.g. the slower mutant protein–substrate pairs of figure 4*a*), and (ii) there was no rapid formation of the first mole of product (i.e. product release was not rate limiting). Thus it was deduced there must be an isomerization of the complex of protonated enzyme–NADH–pyruvate taking 1.5–5 ms before the chemical step in catalysis (Holbrook & Gutfreund, 1973).

A direct measure of shape change seen from a specific point is the change in the ionization of 3-nitro-tyrosine-237 on α-2G as substrate binding induces this helix to approach the acid of Asp 197 on the catalytic histidine loop (Parker *et al.* 1982; Clarke *et al.* 1985). Both these papers originally interpreted the acidification as being due to the approach of glutamate-107. However, Hart (1989) has shown that the α mutant with glutamine at 107 gives the full yellow colour change, whereas in a mutant with neutral asparagine at 197 the colour change is abolished. Thus acidification is due to the approach of Asp 197. The slower of two rates of yellow colour change matches k_{cat} over a wide range of temperatures and solvents.

Useful chemical modification is rare. A more general method is possible: all the natural tryptophan residues are removed from the protein before insertion of a single, fluorescent tryptophan at sites where motion is suspected (the motion of the specificity loop was directly monitored from a tryptophan introduced at the tip of the loop at position 106 and again showed that the maximum velocity of this catalyst was the same as the rate at which the tryptophan probe experienced an environment change in the loop closure process (Atkinson *et al.* 1987; Waldman *et al.* 1988)). The same techniques also enable intermediates during protein-folding to be characterized (Smith *et al.* 1990). In some cases changes in the environment of these probes are faster than the catalytic rate-limiting event and this helps to define the order of secondary structure movements.

DESCRIPTION OF STRUCTURE BEFORE AND AFTER THE SHAPE CHANGE

The fast spectroscopic probes defined the 3 ms shape change around Gly 106, Trp 248 and Cys 165 as being rate-limiting in catalysis; the initial change sensed by Tyr 237 ↔ Asp 197 is ten times faster. During the shape-change induced by binding of saturating concentrations of either the substrate, pyruvate or its inactive analogue, oxamate($H_2N—CO—COO^-$), the fluorescence of the tryptophan residues of the enzyme alters. The rate of this change matches the maximum rate of the enzyme reaction (figure 2*d, e*). The structures before and after the shape change were measured. A single crystal of the pig M_4-enzyme–NADH–oxamate ternary complex was mounted in a microspectrofluorimeter and by observing the fluorescence of the dihydronictinamide ring it was possible to establish that half the oxamate could be washed out by using a buffer containing NADH and ammonium sulphate without disordering the crystal. To obtain the full fluorescence of the pig M_4 LDH and NADH required further washing and the crystal normally cracked. The structures of both the full ternary complex (all subunits containing NADH and oxamate) and of the half binary–half ternary complex were determined by X-ray crystallography to 2.0 Å† (R-factor 0.256) and 2.25 Å (R-factor 0.253) resolution, respectively.

The crystal packing (figure 2*a*) immediately revealed different contacts for two out of the four catalytic vacuoles. Two of the subunits were unrestrained to shape change on oxamate removal and their structures in the ternary (figure 2*f*) and binary (figure 2*g*) states yield a very accurate description of the shape change which accompanies the rate-limiting conversion of binary and ternary complex. The coordinates of the backbones of the two subunits are shown in figure 2*b* superimposed. Figure 2*c* enables the secondary structures which move to be named. Where a shape change has taken place the yellow backbone trace of the binary structure separates from the blue trace of the ternary state.

A sequence of conformational changes starting from the charge balancing when the negatively charged substrate enters the active site can be followed outwards to the surface movements of helices α2G, αD and αH. Tracing the pathway is helped by experimental kinetic information where it is available. At room temperature relating the changes to a substrate-induced trigger is reasonable, but at low temperature and in dimethylsulphoxide it is possible to induce a structure where the first turn-over of the enzyme is 10-times faster than the normal, conformationally restrained k_{cat} (Clarke *et al.* 1985; Atkinson *et al.* 1987). Conserved amino acid residues (picked out as red dots in figure 2*b*), which are at the contact points between the secondary structures that move are listed in table 2. The fastest event is the approach of Asp 197 to Tyr 237 (α2G). The clenching of the specificity loop (98–110) against α2G is slower when seen from a tryptophan inserted at position 106 (Waldman *et al.* 1988). Substrate-binding bridges His 195 and Thr 246, draws in Asp 197 and enables withdrawal of Glu 194 from a hydrophobic pocket formed by Leu 322 and Ile 325 on αH. Movement enables Ser 198 to drag (via an H-bond to Ser 318 on αH) the entire C-terminal helix 1.5 Å in towards the active site. The net result of the changes is a tighter interlocking of the external elements of the active site (the βG/βH turn, the α1G/α2G helix and the active site loop) such that the catalytic groups His 195 and Arg 109 are shielded from the effects of bulk solvent. Many conserved residues occur at the contact points where secondary structures move in the substrate-induced change and in the catalytic centre. Helix αC, which appears remote, donates His 68 to the active site of the *Q*-axis related subunit.

In the ternary complex (figure 2*f*) there are many ordered waters in the catalytic vacuole which form extra stabilizing bridges with the inside surface of the specificity loop and with some of the residues whose

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† $1 Å = 10^{-10} m = 10^{-1} nm$.

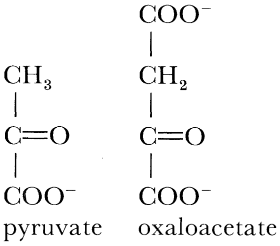
Table 1. *A very active malate dehydrogenase from a lactate dehydrogenase framework* (*from Wilks* et al. *1988*)

enzyme	substrate	k_{cat} / s^{-1}	K_{M} / mM	k_{NADH} / $k_{\mathrm{NADD}(\pm 0.1)}$	$t_{0.5}$90 °C / m	K_{M} / $K_{\mathrm{M}}(+\mathrm{FBP})$	$k_{\mathrm{cat}}/K_{\mathrm{M}}(\mathrm{OAA})$ / $k_{\mathrm{cat}}/K_{\mathrm{M}}(\mathrm{PYR})$
Q102 WT	pyruvate	250	0.06	1.1	7	50	0.95×10^{-3}
	oxaloacetate	6	1.5	2.8	7	n.d.	
R102	pyruvate	0.9	1.8	2.6	6.5	2.8	8.4×10^{3}
	oxaloacetate	250	0.06	1.1	6.5	33	

position alters in the shape change. Fewer low B-value waters are seen in the more solvent-exposed binary complex (figure 2*g*). The strongly ordered H-bonds from these frozen waters to elements of the coenzyme and bound substrate–analogue reduce the number of conformers available along the catalytic pathway to facilitate fast 2-electron transfer over 20 Å (figure 1).

TEST OF ANALYSIS: SYNTHESIS OF A MALATE DEHYDROGENASE ON THE LACTATE DEHYDROGENASE FRAMEWORK

The analysis of specificity suggests only small and singly negatively charged ketoacids enable the specificity loop to close correctly onto α-2G and enable the catalytic Arg 109 to approach the substrate carbonyl and stabilize the transition state by 4–6 kcal mol⁻¹. This analysis was used to suggest designs of an enzyme that would recognize and transform a substrate with two negative charges: that is oxaloacetate instead of pyruvate:



$CH_3{-}C{=}O{-}COO^-$ pyruvate $COO^-{-}CH_2{-}C{=}O{-}COO^-$ oxaloacetate

A fixed-volume vacuole which can only accommodate balanced charges suggests three ways of making the

Table 2. *Conserved amino acid residues in the secondary structure element contacts which move in the binary* (*E-NADH*) *to ternary* (*E-NADH-oxamate*) *conformation change*

(Residues are identical in at least 21 aligned primary structures (J. J. Holbrook, unpublished alignment). This table and figure 2*b*, *c* enable the bonds that transmit conformation information from the surface to the active centre and vice versa to be identified. The coenzyme does not move in this shape change.)

secondary structures	residues	Cα separation binary	ternary	bond type
		Å		
βG–α3G	His 195–Thr 246	14.3	13.9	H-bond via oxamate
βH–βG	Ser 198–Glu 194	6.9	5.9	H-bond
	Asp 197–Glu 194	7.9	7.4	H-bond via water
αH–βH	Ser 318–Ser 198	7.0	5.3	H-bond
	Glu 311–Ser 204	9.8	8.5	H-bond
αH–βD/αD (loop)	Leu 322–Leu 110	9.6	9.1	Hydrophobic
βG–βD/αD	Glu 194–Arg 109	11.5	10.5	H-bond via 2 waters
α1G/α2G–βD/αD	Tyr 237–Arg 109	10.7	10.4	H-bond via water
	Val 232–Arg 109	15.6	12.5	H-bond via 2 waters
βG–α1G/α2G	Asp 197–Tyr 237	10.3	10.3	H-bond
βE/α1F–βG	Asp 143–His 195	6.9	7.0	H-bond
βE/α1F–coenzyme	Asn 140–ribose C_2	4.7	4.6	H-bond
	Val 138–amide C_7	5.7	5.5	H-bond
	Val 138–pyridine N_1	5.8	6.0	Hydrophobic
α2F–coenzyme	Ser 163–amide	6.6	6.5	H-bond via water
	Gly 164–amide	7.0	6.8	H-bond via water
βG–α2F	His 195–Asp 168	7.1	6.8	H-bond
βL–αH	Leu/Val 285–Ala 319	6.0	6.3	Hydrophobic
	Ile/Leu 287–Ala 319	7.3	7.1	Hydrophobic
coenzyme–βD/αD	ribose–Ala 100	6.4	6.2	H-bond
	ribose–Gln 102	6.8	6.5	H-bond via water
coenzyme–βA/αB	pyridine–Val 32	6.1	6.4	Hydrophobic
coenzyme–α3G	pyridine–Ile 250	7.3	7.3	Hydrophobic
α3G–αC (*Q*-axis)	Trp 248–Glu 62	9.3	8.2	H-bond
α2F–αC (*Q*-axis)	Arg 171–His 68	5.2	5.1	H-bond

new activity on this framework (Wilks *et al.* 1988). Increasing vacuole size by changing the bulky Thr 246 to Gly 246 resulted in a greater selectivity for the new target substrate albeit at the expense of a much reduced k_{cat} (loss of H-bond linking His 195 to α-3G). The extra negative charge of the new target substrate was partially balanced by removing non-catalytic negative charge from the periphery of the vacuole (either Asp 197 → Asn or Glu 107 → Gln). Both these mutations shifted selectivity in favour of the new target substrate. Introducing a positively charged amino acid (Gln 102 changed to Arg 102) on the inside surface of the vacuole adjacent to the modelled position of the new target carboxylate had a large effect (table 1). In this construction the old, natural substrate pyruvate cannot provide a counter-ion for the new arginine 102: it is discriminated against by about 950-fold; whereas the new arginine can be solvated by the negatively charged oxaloacetate and is favourably selected by 8400-fold. The net result is a switch against pyruvate and in favour of oxaloacetate by nearly 10^7-fold. Like the wild-type enzyme and substrate, the new framework is limited in rate by a shape change and not by chemistry ($k_H/k_D = 1.1$). In contrast to many previous enzyme redesigns, this new construct is fast (250 s^{-1}) and has a low K_M (60 μM) and unlike natural malate dehydrogenases, the new design is allosterically activated 33-fold by fructose-1,6-bisphosphate.

SYNTHESIS OF A BROAD SPECIFICITY HYDROXYACID DEHYDROGENASE

One use for enzymes is the synthesis of single compound drugs and herbicides. Such drugs make toxicology testing simpler and (since the tragedy of an apparently safe drug for controlling morning sickness being found to contain an enantiomer that impaired foetal development) drug houses are increasingly only bringing forward single enantiomers for approval for human use (de Camp 1989). Natural lactate dehydrogenase makes 100% enantiomeric excess *s*-lactates, but is of limited use in chiral synthesis since most drug intermediates have large side chains that disrupt the fixed volume vacuole. The enzyme is almost unusable with branched side chains (figure 3*b*, solid line). A cross-section of the specificity loop and α2G (figure 2*a*) suggests a wide range of larger pyruvates might be converted to chiral lactates if the 'JAW' region could be made sufficiently hydrophobic and flexible to accommodate a range of branched chain paraffins. In the lower region (α2G) alanines 235–236 were replaced by glycines to both increase the volume for large side chains and to provide conformational flexibility by destabilizing the helix. As can be seen from figure 3*b* this change alone gave only two- to five-fold improvement in k_{cat} for the largest substrates. Making the hydrophilic inside surface of the upper 'JAW' more hydrophobic (Gln–Lys 102–3 to Met–Val) and the introduction of a serine instead of proline-105 to add flexibility also provided only marginal improvements in k_{cat} (for large-scale production of chiral products improved k_{cat} is more important than a low K_M since the high substrate concentration is normally used to aid product isolation). However, when both sets of mutations are made a reasonable α-hydroxyisocaproate dehydrogenase results with a k_{cat} of 18.5 s^{-1} (compared with wild-type k_{cat} of 250 s^{-1} for pyruvate). The improvement was obtained without trial and error. It is unlikely random mutagenesis and selection would produce mutations in two coupled surfaces at once.

A MUTANT WITH ALTERED CONFORMATION CHANGE

In making these new constructions on the lactate dehydrogenase framework the mobile loop was altered and structures with changed rate of the conformation change may have resulted. However, the slower mutants are limited in their maximum rate by the rate of the chemical reduction and show a kinetic deuterium isotope ratio of 2–4 (figure 4*a*). The only experimental information then available is that the shape change (k_{conf}) is faster than the rate of the chemical change (k_{chem}). Most of the results fall in an envelope which increases to 250 s^{-1}. However, for one construction (QKP 102–5 → MVS) the k_{cat} with pyruvate and α-ketobutyrate are slowed to 100 s^{-1}, but without C—H bond breaking becoming rate-limiting (primary deuterium isotope ratio is 1.2). This mutation, which slows the rate of the conformation change involves the inside surface of the mobile specificity loop. The slowing mutation is reversed when the surface against which it closes is made more open when the AA → GG mutation is also recruited. This again suggests the final closure of the specificity loop against α-2G is important in the slowest shape change (250 s^{-1}) and lags in time the fast approach of Asp 197 to Tyr 237 on α2G (estimated at 3000 s^{-1} at 25°; Atkinson *et al.* 1987).

PENALTIES OF A VACUOLE MECHANISM. THE RATE OF THE SUBSTRATE-INDUCED SHAPE CHANGE IS SLOWED BY SOLVENT FRICTION

The method used to prepare the half binary/half ternary crystal shows that if the specificity loop (98–110) movement is prevented then the reordering of α2G and αD and αH are also prevented. Viscous solutions slow the k_{cat} of many enzymes whose rate is conformationally limited and Saburova *et al.* (1988) observed 44% glycerol reduces k_{cat} for pig M_4 lactate dehydrogenase from 350 s^{-1} to 35 s^{-1}. Elements which might transmit the surface frictional drag to the active centre are the conserved bonds between αH and βH (Ser 318–Ser 198, Glu 311–Ser 204) and between αH and αD (Leu 322–Leu 110). Thus in the malate dehydrogenase of *Thermus flavus* which has the same framework as lactate dehydrogenase a spontaneous mutant (Thr 190 to Ile) arose (breaks an H-bond bridge to Thr 315 of α-H) with the k_{cat} increased from 460 s^{-1} to 1200 s^{-1} (Nishiyama *et al.* 1986). An H-bond is also possible between the homologous pair of conserved residues in lactate dehydrogenase (Ser 198 ... Ser 318). This fortuitous mutant suggests it may be unsafe to extend Darwin's ideas on survival of the

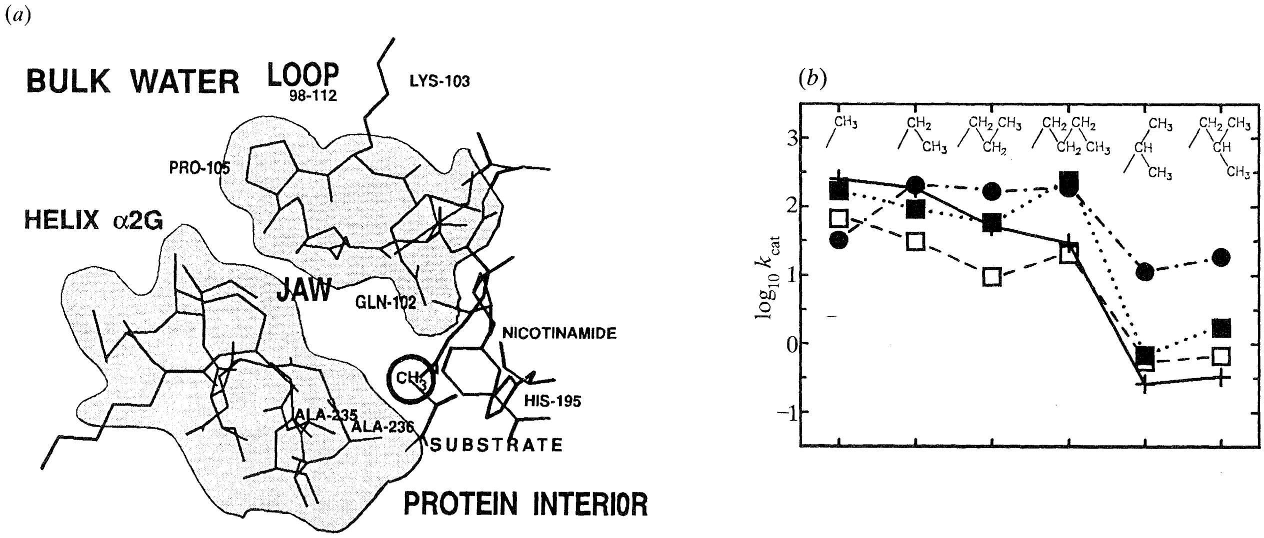


Figure 3. The lactate dehydrogenase catalytic vacuole used to design a broad specificity hydroxyacid dehydrogenase. (*a*) A cross section through the catalytic vacuole showing the two moving surfaces (loop and helixα2G) which seal the vacuole from the solvent. (*b*) A plot of $\log_{10}$ (k_{cat}) for a series pyruvates ($\mathrm{R.CO.COO^-}$) for the wild-type enzyme (—+—), a mutant with $^{235-6}$AA → GG (lower jaw --■--), a mutant with $^{102-105}$QKP → MVS (upper jaw ...□...), and all five mutations at once (-·-·-●-·-·-). Only when both surfaces are changed together is a broad specificity enzyme formed. Replotted from Wilks *et al.* (1990). The fixed size vacuole of the wild-type will not accommodate side chains larger than $\mathrm{—CH_2—CH_3}$.

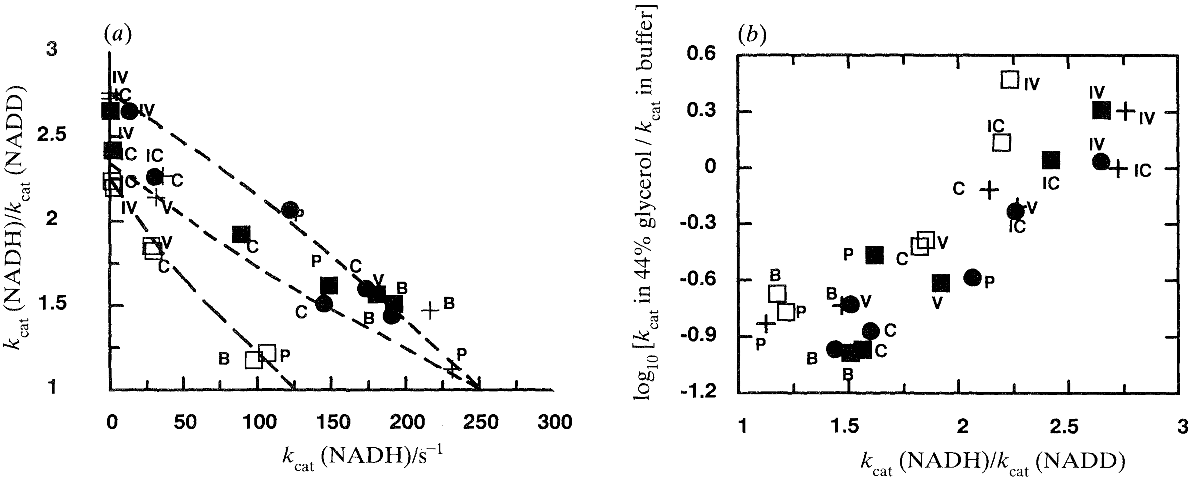
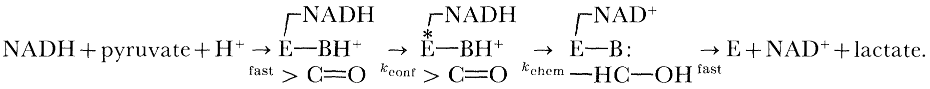


Figure 4. Change in the rate-limiting step from vacuole formation (fast enzyme–substrate pairs) to bond breaking (slow pairs). (*a*) The primary deuterium kinetic isotope ratio as a function of the k_{cat} with NADH for the four enzymes of figure 3*b*: wild-type (+), $^{102-105}$MVS (□), $^{235-236}$GG (■) and all five changes together (●) with P, pyruvate; B, α-ketobutyrate; V, α-ketovalerate, C; α-ketocaproate; IV, α-ketoisovalerate; IC, α-ketoisocaproate.



$$\mathrm{NADH+pyruvate+H^+} \xrightarrow[\text{fast}]{} \begin{matrix}\ulcorner\mathrm{NADH}\\ \mathrm{E{—}BH^+}\\ >\mathrm{C{=}O}\end{matrix} \xrightarrow[k_{\text{conf}}]{} \begin{matrix}\ulcorner\mathrm{NADH}\\ \overset{*}{\mathrm{E}}\mathrm{{—}BH^+}\\ >\mathrm{C{=}O}\end{matrix} \xrightarrow[k_{\text{chem}}]{} \begin{matrix}\ulcorner\mathrm{NAD^+}\\ \mathrm{E{—}B:}\\ \mathrm{—HC{—}OH}\end{matrix} \xrightarrow[\text{fast}]{} \mathrm{E+NAD^+ + lactate.}$$

The lines are drawn according to a reverse reaction mechanism (Holbrook *et al.* 1975), which has two steps after the rapid bimolecular binding of first coenzyme and then substrate: the isomerization of the initially formed ternary complex (k_{conf}) followed by the chemical step (k_{chem}) in which hydride is transferred and NAD$^+$ and lactate are formed on enzyme and then rapidly released into solution. The envelope lines (------) model k_{chem} (NADH)/k_{chem} (NADD) = 2.4–2.8 and $k_{\text{conf}} = 250\ \mathrm{s^{-1}}$ and enclose results for the wild type, $^{235-236}$GG, and the mutant enzyme with all five changes. The line (––––) models the conformational change mutant $^{102-105}$MVS with isotope ratio = 2.3 and $k_{\text{conf}} = 125\ \mathrm{s^{-1}}$. (*b*) The slowing of maximum velocity by 44 % (w/v) glycerol. The decadic logarithm of the ratio of the maximum rate of catalysis of the same constructions and substrates were measured in buffer and in 44 % glycerol. Fast enzyme–substrate pairs where conformation change rate-limits are markedly slowed by glycerol. Slow pairs, where chemistry limits show a slight rate increase. The general trend is visible from 24 enzyme–substrate pairs.

fittest by adaptation (Darwin 1858) to include perfect adaption of individual biomolecules. Lactate dehydrogenase shows improvement of a natural enzyme for an unnatural substrate (see also Clarke *et al.* 1987*b*), but we may now seek and find modest improvement against natural substrates (e.g. the Asp 53 → Ala enzyme uses both NAD^+ and $NADP^+$ (Feeney *et al.* 1989) for cheap NADPH coenzyme regeneration in enzyme reactors).

The effect of bulk viscosity is consistently seen in all the 24 enzyme and substrate pairs of figure 3*b*. Where the framework–substrate pairing gives slow catalysis limited by bond breaking ($k_{H/D} > 1$ in figure 4*a*) the effect of viscosity is small. However, for fast enzyme and substrate pairs limited by the substrate-induced shape change the effect of glycerol is to slow k_{cat} by 5–10-fold. The slowing in glycerol is inversely correlated with the appearance of the primary deuterium isotope effect (figure 4*b*) and strongly supports the concept that, in the natural enzyme, part of the free energy decrease on substrate- or substrate analogue-binding is dissipated in driving the movement of surface peptide structures against friction of the solvent. This system is one of few where description of the coupling of the energy of substrate-binding to the ability of a protein to do work is possible in atomic detail.

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Discussion

A. J. Crumpton (*Fairways, Middle Street, East Harptree, Avon, U.K.*). Professor Blundell alluded to the fact that protein cores were evolutionarily more conserved than the protein surface.

In the final discussion I mentioned that when an active centre was first discovered upon an enzyme it had been necessary to use a non-polar solvent (acetone, free of both water and carbon dioxide, which I had prepared) for the ultraviolet spectroscopy of ribonuclease A.

I asked if any enzymologists present were using non-polar solvents to study the hydrophobic cores of proteins. A few did then report such work.

Plate 1 was printed by George Over Ltd., Rugby, U.K.

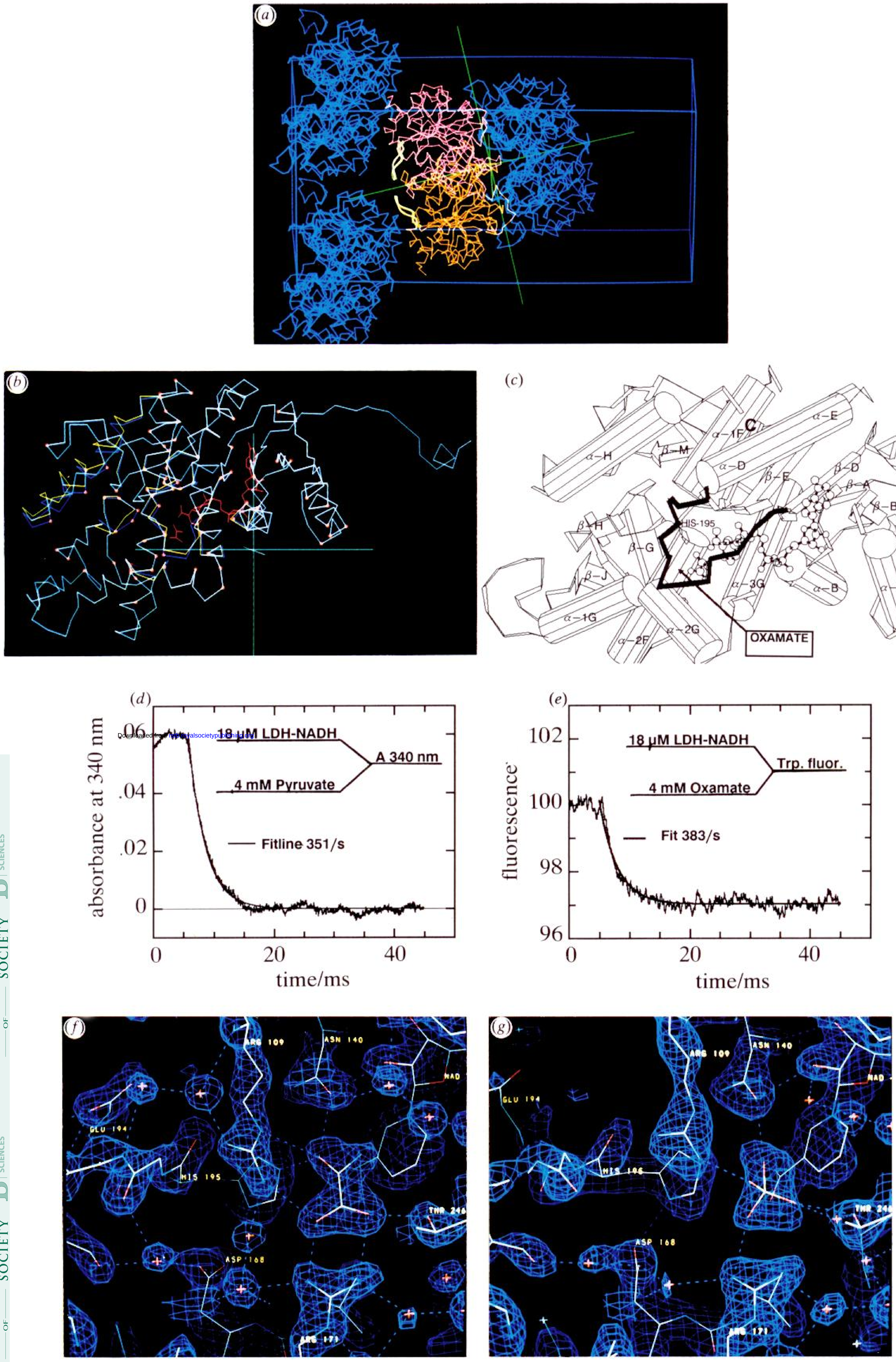

Figure 2. For description see opposite.