Removal of substrate inhibition in a lactate dehydrogenase from human muscle by a single residue change

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Abstract High concentrations of ketoacid substrates inhibit most natural hydroxyacid dehydrogenases due to the formation of an abortive enzyme–NAD^+–ketoacid complex. It was postulated that this substrate inhibition could be eliminated from lactate dehydrogenases if the rate of NAD^+ dissociation could be increased. An analysis of the crystal structure of mammalian LDHs showed that the amide of the nicotinamide cofactor formed a water-bridged hydrogen bond to S163. The LDH of *Plasmodium falciparum* is not inhibited by its substrate and, uniquely, in this enzyme the serine is replaced by a leucine. In the S163L mutant of human LDH-M_4 pyruvate inhibition is, indeed, abolished and the enzyme retains high activity. However, the major contribution to this effect comes from a weakening of the interaction of pyruvate with the enzyme–coenzyme complex.

Key words: Lactate dehydrogenase; Substrate inhibition; Mutagenesis; Enzyme kinetics; Protein modelling

1. Introduction

A common feature of lactate dehydrogenases is their inhibition by excess substrate when converting pyruvate to lactate. The LDH from human muscle is a typical example, while it has a high value of k_{cat} it is strongly inhibited by pyruvate [1]. This greatly reduces its efficiency as a catalyst for the stereospecific reduction of pyruvate derivatives to produce drug synthons in the laboratory or industrial context. The cause of this inhibition is known to be the formation of a covalent adduct between the oxidised forms of substrate and cofactor, i.e. pyruvate and NAD^+ [2]. A natural example lacking substrate inhibition is afforded by the LDH from the malarial parasite, *Plasmodium falciparum* [3]. This protein is unique in the family of lactate dehydrogenases in having a leucine residue at position 163 in place of serine. The hydroxyl group of this serine hydrogen bonds to the nicotinamide amide of NADH, via a water molecule, in all other known LDH crystal structures [4]. Inspection of a model of *pf*LDH derived from the gene sequence and known LDH structures suggests that the loss of a good interaction between residue 163 and the nicotinamide ring might be, at least in part, responsible for the loss of substrate inhibition by reducing the productive binding of the oxidised cofactor, NAD^+ [5]. An engineered example is known in the case of D-LDH from *Lactobacillus delbrueckii* subsp. *bulgaricus* where removal of substrate inhibition occurs when histidine-205 is replaced by glutamine, thus losing an ionic contribution to cofactor binding via the pyrophosphate group [6].

In this paper we describe the modelling and preparation of two variants of hM_4LDH, replacing the serine at position 163 by alanine and leucine, and we compare the kinetic and binding behaviours of these proteins with the wild type. The S163L mutant showed no detectable substrate inhibition, but the cause for this is not simply due to reduced NAD^+ binding.

2. Materials and methods

2.1. Molecular modelling

In the absence of a human LDH crystal structure the coordinates of the pig structure were used for modelling as the sequence of amino acids differs in only 23 positions on the surface of the protein and remote from the active site. A single subunit of the ternary complex of pigM_4-LDH with NADH and oxamate [7] was set up for calculation in the following manner. The first 19 residues of the N-terminus were deleted as these pack against a second subunit, the oxamate was converted to pyruvate, hydrogen atoms were added consistent with pH 7.0 and extra water molecules added to a depth of 5 Å over the surface. Residue replacement of serine-163 generated the alanine and leucine mutants. Simulations were carried out with and without the bridging water (number 1H in the X-ray structure) in the alanine mutant and without this water in the leucine case. Each structure was relaxed by energy minimisation using a 10 Å non-bonded cutoff under the following protocol. Three sets of 200 cycles of template forcing to the initial coordinates with restraints of 50, 20 and 5 kcal/Å, respectively, then unconstrained minimisation until the average absolute derivative fell below 0.01 kcal/Å. Structure manipulation and inspection was carried out using InsightII 2.35 and energy calculations with Discover 2.95 (Biosym Technologies Inc.) using the cvff forcefield [8].

2.2. Chemicals and growth media

Agarose, low-melting-point agarose, ethidium-bromide and sodium pyruvate were from Sigma. NADH disodium salt was from Boehringer Corp. Other reagents were from BDH. The *Escherichia coli* strains were cultured in double strength (2×) YT broth (1% yeast extract, 1.6% tryptone, 0.5% NaCl) or grown on 2×YT agar plates. For competent cells the TG2 strain of *E. coli* was grown on minimal plate (containing 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.1% NH_4Cl, 0.05% NaCl and 1.5% agar).

2.3. Genetic manipulation procedures

Restriction endonucleases and DNA ligases were purchased from Boehringer Corp. and used according to the manufacturer's instructions. Isolation of plasmid DNA, preparation of DNA fragments, ligation and transformation of *E. coli* cells were carried out according to Sambrook et al. [9].

The oligonucleotide site-directed mutagenesis was generated by the PCR-based overlap extension method [10] using a Perkin Elmer DNA thermal cycler. The template used for the mutagenesis was pHLDHA22 [11] containing human LDH-A (muscle) cDNA purified from *E. coli* TG2 cells that were used as a host for transformation and

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Abbreviations: LDH, lactate dehydrogenase; *pf*, *Plasmodium falciparum*; hM_4, human M_4; *Taq*, *Thermus aquaticus*; *Pfu*, *Pyrococcus furiosis*; PCR, polymerase chain reaction

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expression of human *LDH* genes in plasmids pKK223-3 and pUC-18 (Pharmacia P.L.).

Oligonucleotides 5′-GTG/TTA/TTG/GAC/TGG/GTT/GCA/ATC-3′ and 5′-CTG/TTA/TTG/GAG/CTG/GTT/GCA/ATC-3′ were used for the site-directed mutagenesis of the cDNA coding for the human *LDH-A* gene to give proteins S163L and S163A, respectively (nucleotides in bold type show the mutant regions). These were synthesized using a Perseptive Biosystems Expedite DNA synthesizer.

The conditions of the mutagenesis used to generate the two overlapping fragments were identical (20 ng of template DNA, 40 ng of each primer and unit DNA polymerase in 50 μl total volume) and were tested using *Taq* polymerase. The final mutagenesis was carried out using *Pfu* polymerase which has proof reading activity. In the first stage N- and C-terminal fragments were obtained using the following parameters: 94°C for 1 min, 50°C for 1.5 min, 72°C for 2 min for 20 cycles. The PCR products were run on a 0.8% low-melting-point (LMP) agarose gel, the ~1.7 kb bands were cut out and purified using phenol extraction. Each purified fragment was added to a subsequent PCR reaction where they were overlapped and extended, then amplified during the third reaction. The conditions for the second reaction were: 94°C for 2 min, 50°C for 1 min, 72°C for 2 min for 7 cycles and for the third: 94°C for 1.5 min, 50°C for 1 min, 72°C for 3 min for 20 cycles.

2.4. Nucleotide sequencing

Nucleotide sequence determinations were made by the chain termination method of Sanger et al. [12] using the Sequenase version 2.0 DNA sequencing Kit from USB and autoradiography detection (S^{35}-labelled dATP used was from Amersham International plc., Cleveland, OH).

2.5. Purification of wild-type and mutant hLDH proteins from E. coli

Cells grown in 2×YT broth (containing Ampicillin) were harvested by centrifugation (4000×*g*, 20 min) and were resuspended in minimum volume of 50 mM TEA (triethanolamine hydrochloride) at pH 6 then lysed by sonication on ice (5×15 s at power level 7 using a Model XL 2010 sonicator from Heat Systems Ultrasonics, Farmingdale, NY). After the cell debris was removed by centrifugation (15000×*g*, 30 min) the enzymes were precipitated by adding 40 g $(NH_4)_2SO_4$/100 ml of the supernatant and left for 1 h on ice. The enzymes were recovered by centrifugation (15000×*g*, 20 min). The pellet was dissolved in a minimum volume of 50 mM TEA buffer (pH 6) and dialysed against 50 vol. of that buffer overnight at 4°C. The dehydrogenases were purified by affinity chromatography on a 3×12 cm column of oxamate–Sepharose pre-washed with 0.5 mM NADH. The enzymes were added to the column in 10 mM NADH and eluted with 50 mM TEA at pH 9 containing 1 M NaCl then stored as the precipitate formed when 40 g $(NH_4)_2SO_4$/100 ml of eluate was added. The S163L mutant gave much better chromatographic separation when the column was pre-washed with 10 mM NADH in 10 mM TEA buffer at pH 6. Enzyme content of the eluates was estimated from the intensity of Coomassie Blue stains after electrophoresis on polyacrylamide gel.

2.6. Steady-state kinetics

The enzyme precipitates from the purification were pelleted by centrifugation (15000×*g*, 20 min) then resuspended in minimum volume of 20 mM bis-Tris-50 mM KCl buffer (pH 6) and dialysed against 100 vol. of this buffer overnight at 4°C. Protein concentrations of the purified enzymes were determined measuring the absorbance at 280 nm using absorbance values of 1.20 for 1.0 mg/ml LDH-M_4. These values were calculated from the gene-derived contents of tryptophan and tyrosine of the protein subunits. The steady-state kinetic measurements were made by following the change in absorbance at 340 nm (366 nm at higher pyruvate (>30 mM) concentrations) during the NADH to NAD^+ conversion at 25°C. The initial concentration of NADH was 200 μM and reaction was initiated by addition of pyruvate. The values of K_m for NAD^+ were measured in steady-state initial velocity experiments at pH 9 (50 mM glycine) and 0.2 mM L-lactate. Kinetic constants were evaluated using Grafit version 3 (Erithacus Software, Staines, UK) using $v=v_{max}[S]/([S]+K_M+[S]^2/K_i)$.

2.7. Measurements of enzyme activity in rapid mixing experiments

Measurements of the steady-state rate over a time period of 0.2 s (too short to allow build up of the abortive ternary complex) were made using an SF 51 stopped-flow apparatus (Hi-Tech, Salisbury, Wiltshire, UK). One volume of enzyme solution was mixed with one volume of an NADH/pyruvate mixture and the decrease in absorbance was recorded as the NADH was oxidized to NAD^+. After mixing, the enzyme concentration was 0.3 mg/ml and NADH 0.2 mM. Experiments were performed with pyruvate concentrations of 0.05 mM, 0.1 mM, 0.2 mM and 0.4 mM. The buffer contained 20 mM bis-Tris/50 mM KCl, pH 6.

2.8. Measuring binding constants by tryptophan quenching

The dissociation constants of NADH and NAD^+ were measured from the decrease in fluorescence of enzyme tryptophan on complex formation. Excitation was at 285 nm and emission at 340 nm using a Perkin Elmer spectrofluorimeter. For each experiment 2.0 ml of a freshly made 2 μM solution of the LDH apoenzyme was used. The concentrations of ligand stock solutions were 1 mM NADH and 0.5 M NAD^+. Each stock solution was added in 2 μl aliquots during the titrations. For the NADH titrations the binding curves were fitted to a tight ligand binding equation and corrected for the inner filter effect of NADH:

$$\text{signal} = SX-(K+E+L-\sqrt{((K+E+L)^2-4*E*L)}/2*E)*(SX-SN)$$

where SX is the initial fluorescence intensity, SN the final intensity, K is the dissociation constant, E the concentration of enzyme active sites and L the concentration of nucleotide. For the NAD^+ titrations the binding curves were similarly corrected for the inner filter effect and fitted to the weak ligand binding equation:

$$\text{signal} = SX-(L*(SX-SN)/(K+L))$$

In a similar fashion, the dissociation constants of oxamate from the E:NADH complexes were measured from the decrease of the fluorescence of bound NADH. Excitation was at 340 nm and emission measured at 435 nm. For each experiment 2.0 ml of a freshly made solution of an equimolar mixture of NADH and LDH. The concentration of protein used was 15 μM in the case of S163L and 2 μM in the case of S163A and wild type. Titrations were performed by adding 2 μl aliquots of 0.8 M oxamate to the S163L:NADH solution, and 2 μl aliquots of 10 mM oxamate to the S163A:NADH and WT:NADH solutions. K_d values were obtained by fitting the experimental titration curves to the weak ligand binding equation described above.

3. Results and discussion

3.1. Mutagenesis

Two primers, each 24 nucleotides long, were designed to

Table 1
Kinetic and binding constants for the wild-type and mutant proteins

Protein (μM)	k_{cat}	K_M pyr (s^{-1})	K_i (mM)	K_d oxamate (mM)	K_d NADH (μM)	K_d NAD^+ (μM)	K_M NAD^{+a} (μM)
Wild type	260 ± 3	0.158 ± 0.006	3.9 ± 0.7	26 ± 3	0.62 ± 0.05	198 ± 16	102 ± 10
S163A	168 ± 13	0.249 ± 0.049	4.7 ± 0.9	49 ± 5	0.94 ± 0.06	172 ± 7	324 ± 7
S163L	119 ± 3	6.5 ± 1.0	none	3100 ± 100	5.6 ± 0.6	230 ± 15	347 ± 7

Conditions are described in Section 2.
[a]Apparent K_M at 0.2 mM lactate, pH 9.

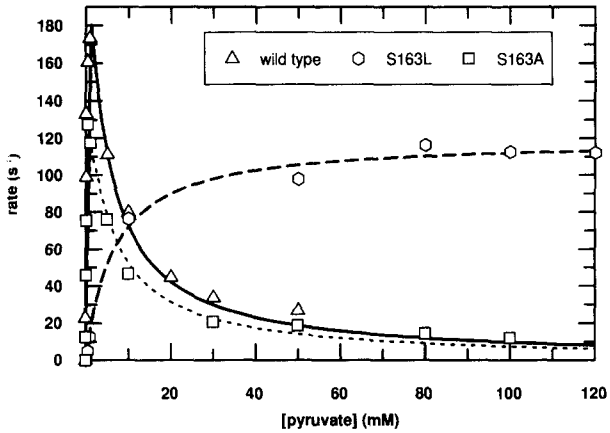


Fig. 1. Steady-state kinetic behaviour of wild-type, S163A and S163L mutants of human LDH-M_4 obtained by monitoring the decrease in absorbance at 340 nm. Catalytic rate constants are given in moles substrate consumed per mole active site per second. The calculated kinetic constants corresponding to the fitted curves are in given in Table 1.

after the cDNA coding for the A isoform of the human *LDH* gene to replace serine-163 in the M_4 protein by alanine or by leucine. Site-directed mutagenesis using the pHLDHA22 plasmid was carried out, the products ligated into pKK223-3 and transformed into TG2 host cells as described in Section 2. The two mutant genes were each sequenced in the region coding for amino acid 163 and showed only the required changes to the published sequence [13]. The transformed bacteria all strongly over produced their respective LDH proteins. The purified enzymes were all greater than 95% pure as judged by polyacrylamide gel electrophoresis in SDS.

2. Kinetics and binding experiments

The steady-state kinetic data of the wild-type and alanine mutant shown in Fig. 1 can, in principle, be fitted to give the three kinetic constants k_{cat}, K_M (pyruvate) and K_i. In practice these constants are so strongly coupled it is difficult to obtain a reliable fit. For this reason, stopped-flow kinetic measurements were carried out to determine k_{cat} and K_M (pyruvate) before the onset of substrate inhibition. The E:NAD$^+$:pyruvate complex is a slowly formed species and requires 1–2 s to accumulate detectably in these conditions (see Fig. 2). These data were used, in turn, to fit the steady-state kinetic curves and so determine reliable K_i values. Fitting the data of the leucine mutant to these three constants gave a K_i value greater than 1 M, i.e. substrate inhibition is undetectable in this protein. Binding affinities for the proteins with NADH and NAD$^+$ were determined by titration of protein fluorescence emission. Binding affinities for the binary complexes of the proteins and NADH with oxamate were determined by titration on the NADH fluorescense emission. All these results are shown in Table 1.

3. Suppression of substrate inhibition

In the absence of crystal structures molecular modelling can provide valuable insights into possible structural and energetic consequences of point mutations in proteins. Superimpostion of the Cα positions of the four minimised ternary complexes in the six possible permutations resulted in RMS deviations of the Cα positions lying between 0.30 and 0.45 Å. These low values imply that the two mutations have little effect on the overall structures of the proteins. Fig. 3 shows the corresponding structures around position 163. All the complexes exhibit catalytically competent geometry with the A-side hydride of NADH in close contact with the carbonyl carbon of pyruvate. Modelling the S163A complex with and without an intervening water molecule gives geometries closely similar to wild type. For example, the nicotinamide amide carbon to 163 Cα distance for S163A with an intervening water (Fig. 3b) is 0.2 Å longer than that in wild type (Fig. 3a), and 0.2 Å shorter when modelled without the water (Fig. 3c). Despite the loss of hydrogen bonding between NADH and residue 163, the K_d for NADH of the S163A mutant is only 1.5 times higher than that of the wild type and NADH. This may be due to a trade off in the wild-type complex between favourable enthalpy from hydrogen bonding and unfavourable entropy caused by sequestering a water molecule from the solvent. This, in turn, implies that the model of S163A without an intervening water (Fig. 3c) is correct and that the principle role of serine-163 in LDHs is to position the nicotinamide ring for reaction, rather than to contribute towards the total NADH binding energy. Similar behaviour is shown by the S163A mutant of a bacterial protein [15]. A greater change in geometry occurred in the S163L complex due to the larger bulk of this hydrophobic residue. In this case, the nicotinamide amide carbon to 163 Cα distance is predicted to be 0.5 Å longer than in wild type. The nicotinamide amide has also rotated out of the plane of the ring by 60°. This disruption is reflected in a K_d value for the interaction between S163L and NADH (Table 1) which is 10-fold higher than wild type. This corresponds to a loss of about half the contribution usually made by the dihydronicotinamide group towards NADH binding in LDHs [14].

In contrast with the NADH binding results, the NAD$^+$ K_d

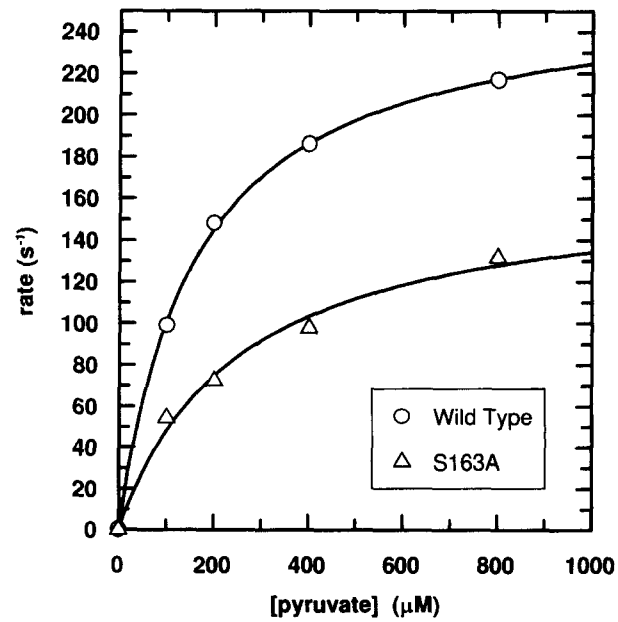


Fig. 2. Determination of steady-state kinetic parameters by stopped flow rapid-mixing methods for wild-type and S163A proteins. The formation of the inhibitory NAD$^+$/pyruvate adduct is slow and does not occur to any measurable extent during the first 100 ms of the enzyme catalysed reaction. The rates are the initial velocities given in moles substrate consumed per mole active site per second measured over the first 50 ms of reaction.

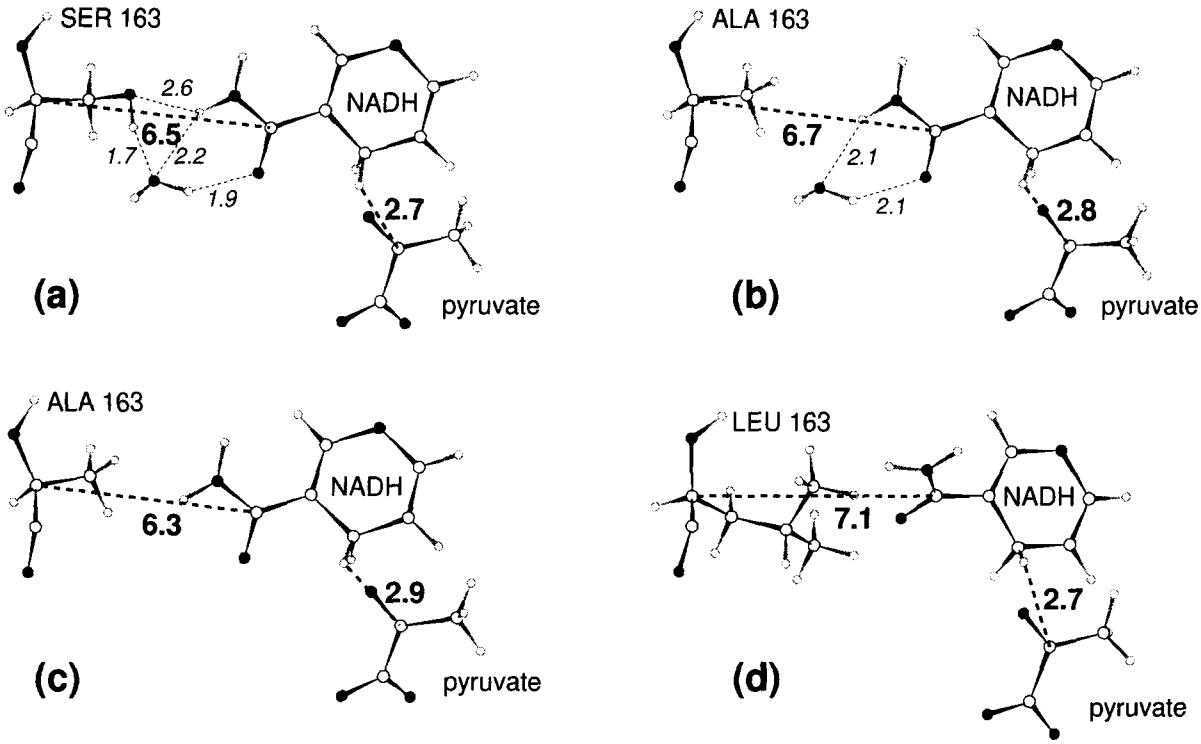
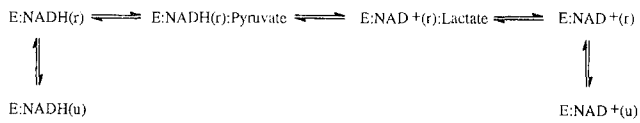


Fig. 3. Geometries around residue 163 in the energy minimised ternary complexes. ○, Carbon and hydrogen atoms; ●, nitrogen and oxygen. Hydrogen bonds are shown as light dashed lines and distances in italics. Other distances are in bold dashed lines and bold text. (a) Wild type; (b) S163A with intervening water; (c) S163A without intervening water; (d) S163L.

values for all three proteins are very similar, all lying around 200 μM. This observation is consistent with previous work which showed that the oxidised nicotinamide group contributes nothing towards the overall binding of NAD^+ to LDHs, in fact the binding of NAD^+ is weaker than adenosine-diphosphate–ribose. In the light of these observations the complete loss of substrate inhibition in S163L might, at first sight, be surprising. However, inspection of Table 1 indicates that the reason that substrate inhibition has been lost is a consequence of the much weaker binding of pyruvate in this mutant. This weakening of pyruvate binding is inferred from the 40-fold increase in K_M for pyruvate by S163L and also by the 120-fold increase in K_d for oxamate by S163L:NADH compared with the wild type:NADH. Hence it must be weakening of pyruvate binding rather than weakening of NAD^+ binding which results in no significant formation of the inhibitory adduct between pyruvate and S163L:NAD^+ under the reaction conditions studied.

3.4. Mechanistic interpretation

The striking feature of the S163L mutation is that although the residue makes contact with the nicotinamide group its most potent effect is on substrate affinity rather than coenzyme binding or catalytic rate. The simplest explanation requires the postulation of two forms of binary complex between NADH or NAD^+ and enzyme and also a consideration of the nature of an obligatory order reaction. The fact that LDH must bind the coenzyme first demonstrates that the substrate binding site is only formed after establishing a receptive binary complex. The 'receptive' binary complex (r) is postulated to have geometry similar to that seen in crystal structures and can bind pyruvate tightly in the normal manner. The 'unreceptive' forms (u) have a deformed geometry and cannot bind pyruvate as shown in the scheme below:



This deformed geometry is most likely characterised by perturbations of the nicotinamide ring from its orientation in the wild-type complexes, due to blocking of the nicotinamide amide pocket by the inserted leucine-163. The identification by NMR of a mixture of LDH/NAD^+ binary complexes of this kind has been reported [16]. Although the equilibrium between E:NADH(r) and E:NADH(u) in the mutant is heavily in favour of the latter, the binding of pyruvate can induce the receptive state at the cost of net binding energy. Under these conditions the observed K_M for pyruvate will approximate to the product of the K_M of E:NADH(r) and its fractional population, but the observed k_{cat} will just reflect the effectiveness of the ternary complex to proceed to products.

This model accounts for the behaviour of both mutant proteins in the following way. Suppose that both wild type and S163A bind NADH almost exclusively in the receptive geometry. Then the 1.5-fold weaker binding of NADH shown by S163A is echoed in a 1.6-fold raising of the K_M for pyruvate, a 1.6-fold lowering of k_{cat} and a 1.2-fold raising of K_i. Suppose that only about 1% of the binary complex of S163L and NADH is in the receptive form and the rest is unreceptive. The fact that there is only a 10-fold increase in K_d for NADH relative to wild type suggests that the dihydronicotinamide group in the unreceptive form still contributes to NADH binding as this group usually contributes by a factor between

10^2 and 10^3 [14]. The receptive form can bind pyruvate and the enzyme reaction proceeds with a 2-fold reduction in k_{cat} due to a slightly poorer geometry of the catalytic complex than that of wild type. However, the fact that only 1% of the binary complex is in the receptive form results in a 100-fold increase in the observed K_M of S163L for pyruvate. If the geometric requirements for forming the abortive E:NAD^+:pyruvate complex are similar to those for formation of E:NADH(p):pyruvate then a similar proportion of receptive and unreceptive complexes of S163L with NAD^+ would raise K_i to at least 0.4 M.

4. Conclusions

The role of serine-163 in lactate dehydrogenases appears to be one of positioning the nicotinamide group of the cofactor rather than contributing significantly towards binding. Hence, in the human protein replacing serine-163 by alanine has a small effect upon the kinetic properties of the enzyme with respect to wild type. Replacement of serine-163 with the larger leucine residue, however, has a pronounced effect upon the kinetic properties of the protein, while the k_{cat} of this mutant is reduced by only 50%, substrate inhibition is completely removed. This effect cannot be simply explained by a weakening of NAD^+ binding leading to a reduction in the rate of abortive complex formation, since the K_d for NAD^+ in this mutant is similar to wild type. These effects, along with the 10-fold increase in K_d for NADH and the 40-fold increase in K_M for pyruvate are best explained by a model in which the binding energy of pyruvate is used to re-align the nicotinamide group of the cofactor thus increasing both K_M and K_i for this substrate while leaving k_{cat} largely unaltered.

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