Vol. 146, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1987 Pages 346-353

THE IMPORTANCE OF ARGININE 171 IN SUBSTRATE BINDING BY *BACILLUS STEAROTHERMOPHILUS* LACTATE DEHYDROGENASE

Keith W. Hart[a], Anthony R. Clarke[a], Dale B. Wigley[a], William N. Chia[a], David A. Barstow[b], Tony Atkinson[b], and J. John Holbrook[a]

[a] Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol (U.K.) BS8 1TD

[b] PHLS Centre for Applied Microbiology and Research, Microbial Technology Laboratory, Porton Down, Salisbury, Wiltshire (U.K.) SP4 OJG

Received June 2, 1987

A variant of lactate dehydrogenase from *Bacillus stearothermophilus* has been engineered by site-directed mutagenesis in which an active-site arginine residue at position 171 in the protein sequence is replaced by lysine. Replacement of this arginine by lysine has no effect on co-enzyme binding, a relatively small effect on the rate of turnover of the enzyme, but causes a 2000-fold increase in the Michaelis constant for pyruvate, a 6000-fold increase in the dissociation constant for oxamate and results in a Michaelis constant for lactate which is too high to measure. The decrease in binding energy for these carboxylate-containing substrates caused by this mutation is very large, around 5.5 kcal.mol^{-1} and in part, is explained by

ABBREVIATIONS: LDH , Lactate dehydrogenase; FBP , fructose-1, 6-bisphosphate

0006-291X/87 $1.50

the small increase in the distance of a lysine-substrate carboxylate interaction at this site and the absence of the additional hydrogen bond from a two-point arginine-carboxylate interaction. Consistent with this last observation, the ability of this mutant enzyme to stabilize an NAD^+-sulphite compound in its active site (an alternative enzyme-substrate complex which does not involve bifurcated bonding to arginine) is only reduced 14-fold.

X-ray structures of enzyme-coenzyme-substrate complexes of the NAD-dependent L-lactate dehydrogenases the substrate carboxylate group is bonded to the guanidinium group of arginine 171, a residue conserved in all known LDH protein sequences (1,2). The bonding arrangement is co-planar and bifurcated and, in an enzyme which has only two attachment points to the substrate (the other being a hydrogen bond to histidine-195), we wished to evaluate its energetic contribution to substrate binding. To do this we have replaced arginine 171 with lysine, an amino acid of similar bulk and with the same charge, but which can only form a single-point interaction with the carboxylate group of the substrate.

MATERIALS AND METHODS

Preparation of mutant protein. The amino acid substitution was made by site-directed mutagenesis of the LDH gene in M13, as described by Winter *et al.*(3). The entire gene was sequenced to ensure that only the directed mutation had arisen and was expressed in the pKK223-3 plasmid in *E.coli*, as we have previously described (4). Over-expressing *E.coli* cells were grown overnight (16 hrs) in a 2xYT medium containing ampicillin and were harvested by centrifugation (5 mins at 4,000 x g). The cell pellet was resuspended in 10 mM triethanolamine

hydrochloride/NaOH pH 6.0 and the cells lysed by sonication. The cell debris was removed by centrifugation (20 mins at 10,000 x g) and the supernatant precipitated by the addition of ammonium sulphate (430 g per litre). The precipitated protein was spun down (20 mins at 10,000 x g), resuspended in and dialysed against the above buffer for 16 hrs at 4 °C (3g per litre of charcoal was added to the dialysis buffer). The protein solution was spun (20 mins at 10,000 x g) to remove any precipitated protein and loaded onto a column (2.5 x 19 cm) of Cibacron Blue-Sepharose gel (F3GA (5)) equilibrated in the above buffer (the poor substrate-binding ability of the mutant enzyme precludes the affinity-gel purification devised for the wild-type enzyme (6)). The column was washed extensively with the same buffer and the LDH eluted using 1mM NADH. The fractions containing LDH activity were loaded onto a column (2 x 10 cm) of DEAE-sepharose (CL-6B) equilibrated in the above buffer, washed, and eluted on a salt gradient (0.1-0.3M NaCl). The LDH peak eluted at about 0.2M NaCl and was greater than 98% pure as judged by polyacrylamide gel electrophoresis in SDS (7).

RESULTS

(i) The effect of mutation on substrate binding and catalysis.

The main influence of the arginine for lysine substitution on the catalytic properties of the enzyme is a large weakening of substrate-binding (see Table I). The K_m for pyruvate is increased by a factor of 2000 and the K_m for lactate becomes too high to measure. Similarly the dissociation constant for oxamate (an isosteric and isoelectronic analogue of pyruvate) which is raised from 0.03 mM to 200 mM. The k_{cat} for pyruvate has been reduced less significantly (by a factor of 14). The k_{cat} for lactate cannot be measured because the binding of lactate is too weak .

Table I

PROPERTIES OF THE LYSINE-171 MUTANT OF *B.STEAROTHERMOPHILUS* LDH

PROPERTY	CONDITIONS	NATIVE (ARG 171)	MUTANT (LYS 171)
1. Oxamate			
K_d	5 mM FBP	30 μM	200 mM
2. Pyruvate			
K_m	50 mM FBP	60 μM	125 mM
k_{cat}	5 mM FBP	250 s^{-1}	18 s^{-1}
3. Lactate			
K_m	5 mM FBP	50 mM	>1 M
k_{cat}	"	4 s^{-1}	>0.2 s^{-1}
4. NADH			
K_d	"	1.5 μM	2.5 μM
5. NAD^+			
K_d	"	100 μM	90 μM
6. K_d $SO_3^=$	"	4 μM	56 μM
7. Thermal Stability	"	7mins	2mins
8. FBP activation		50	41

Unless stated, all parameters were measured at 25°C in 100mM triethanolamine/HCl at pH 6.0. 1. Determined fluorometrically (12). 2. Determined in assay solution containing saturating (0.2mM) NADH. 3.Determined in assay solution (pH 8.0) containing saturating (2mM) NAD^+. 4. Measured from NADH fluorescence anisotropy (12). 5. Measured by competition with NADH binding (measured as above). 6. $K_{d(app)}$ at pH 7.2, 15μM sites, 0.5mM NAD^+. Monitored by absorbance increase at 327 nm. 7. Thermal stability was measured by incubating the 2μM enzyme subunits at 90°C in the presence of 5mM FBP and assaying the residual enzyme activity. 8. FBP activation is given as the ratio of the rate of catalysis (at a concentration of pyruvate that is 10% of the activated K_m : 6 μM for Arg-171; 12.5 mM for Lys-171) before and after addition of 5mM FBP i.e activated rate / unactivated rate.

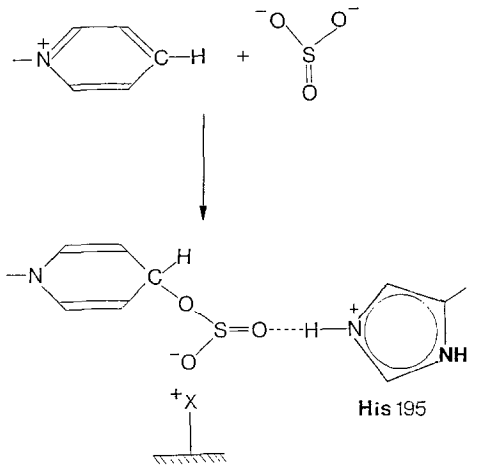


Figure 1. Stabilization of the NAD-sulphite adduct in the enzyme active site. X^+ is either arginine-171 or lysine-171.

(ii) The effect of the mutation on coenzyme binding and thermal stability.

The dissociation constants for both NADH and NAD^+ are unaffected by this mutation and the thermal stability is altered only slightly (see Table I). These observations suggest that the general structure of the enzyme has not been disrupted and that the influence of the mutation is local.

(iii) The formation and stabilization of the NAD-sulphite complex in the active site of the enzyme.

Lactate dehydrogenases are able to catalyse the addition of the sulphite dianion to enzyme-bound NAD^+ and stabilize the product in the active site. This reaction (see Fig.1) depends on the ability of the enzyme (a) to bind NAD^+ and sulphite in an orientation productive for a nucleophilic attack on the C4 of the coenzyme by an oxy-anion of $SO_3^=$, (b) to bind the neutral coenzyme ring of the product tightly to give a stable "on-enzyme" complex and on histidine 195 being protonated (8). Table I shows that, in contrast to binding natural carboxylate-containing substrates, the apparent K_d for $SO_3^=$ in the above reaction is

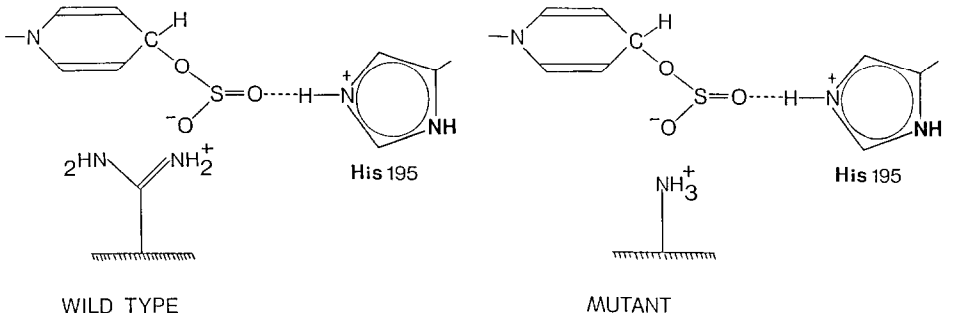
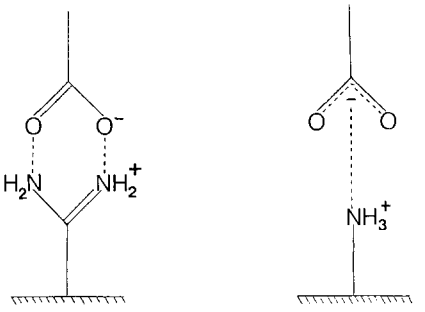


Figure 2. A schematic comparison of the ability of an arginine (wild type) and a lysine (mutant) residue at position 171 to stabilize enzyme-bound NAD-sulphite.

relatively unchanged by the mutation. Figure 2 suggests that lysine and arginine are equally capable of stabilizing the oxy-anion of NAD-sulphite (NAD-SO_3^-) as this requires only a point positive charge. Thus in the interaction between NAD-sulphite and the active site there should be little difference between a lysine and an arginine at this locus. The observed weakening by a factor of 14 may reflect purely the loss of binding energy which results from the expected increase (0.1nm) in charge separation in a lysine-carboxylate over an arginine-carboxylate pair.

DISCUSSION

The replacement of arginine with lysine in the active-site of this lactate dehydrogenase is fairly specific in its effect: it weakens the binding of substrates greatly but has only a small effect on turnover rate, coenzyme binding, FBP activation, thermal stability and sulphite binding. This indicates that the folding of the mutant protein is essentially the same as that of native and that the effect of mutating this residue is restricted to its direct interaction with the carboxylate group of the substrate. The results show that the difference in binding



WILD TYPE MUTANT

Figure 3. The difference in bonding between an arginine-carboxylate interaction (wild type) and a lysine-carboxylate interaction (mutant).

energy between a carboxylate-lysine and carboxylate-arginine bond is extremely large (5-6 kcal.mol^{-1}).

To account for this we suggest three factors. Firstly, the lysine side-chain is shorter and thus the charge-charge interaction with the carboxylate group may be more distant in the mutant than in the wild type enzyme. In the closely homologous mammalian crystal structures the arginine-carboxylate charge separation is 2.5 Å. However, with an extended lysine side chain this distance would be increased to 3.5 Å and taking a value of 20-40 for the local dielectric constant there would be a loss of 1-2 kcal.mol^{-1} of binding energy in the mutant. Secondly, there is a two-point interaction available in the arginine-carboxylate pairing and only a one-point in the lysine-carboxylate. Hence the wild-type enzyme has one hydrogen bond more in addition to a charge-charge interaction (see Fig.3). Finally, there is a large difference between the hydrophilicity of lysine and of arginine; lysine has a side-chain hydration potential of -9.5 kcal.mol^{-1} and arginine -20 kcal.mol^{-1}(9). This is due to the greater number of electronegative atoms in the

guanidinium group and hence its greater capacity for binding water molecules. Given that the binding of substrate in the aqueous active site of LDH will be largely entropy driven (10,11) then it is likely that the formation of a carboxylate-arginine bond will displace a greater number of water molecules to the bulk phase than will a carboxylate-lysine. This entropic factor may be very important in the high stability of the ionic bond in the wild-type enzyme.

ACKNOWLEDGMENTS: We thank the SERC for a project grant and personal fellowship (A.R.C.).

REFERENCES

(1) Grau,U.M., Trommer,W.E. and Rossmann,M.G. (1981) J. Molec. Biol. *151*, 289-307.
(2) Smiley,I.E., Koekoek,R., Adams,M.J. and Rossmann,M.G. (1971) J. Molec. Biol. *55*, 467-475.
(3) Winter,G., Fersht,A.R., Wilkinson,A.J., Zoller,M. and Smith,M. (1982) Nature *299*, 756-758.
(4) Barstow,D.A., Clarke,A.R., Chia.W.N., Wigley,D.B., Sharman,A.S., Holbrook,J.J., Atkinson,T. and Minton,N.T. (1986) Gene *46*, 47-55.
(5) Thompson,S.T., Cass,K.H. and Stellwagen,E. (1975) Proc. Natl. Acad. Sci. USA. *72*, 669-672.
(6) Clarke,A.R., Waldman,A.D.B., Munro,I. and Holbrook,J.J. (1985) Biochim. Biophys. Acta *828*, 375-379.
(7) Laemmli,U.K. (1970) Nature *227*, 680-685.
(8) Parker,D.M., Lodola,A. and Holbrook,J.J. (1978) Biochem.J. *173*, 959-967.
(9) Wolfenden,R., Anderson,L., Cullis,P.M. and Southgate,C.C.B. (1981) Biochemistry *20*, 849-855.
(10) Fersht,A.R. (1985) "Enzyme Structure and Mechanism", Freeman, New York, Ch. 2.
(11) Jencks,W.P. (1969) "Catalysis in Chemistry and Enzymology". McGraw-Hill, New York.
(12) Clarke,A.R., Atkinson,T., Campbell,J.W. and Holbrook,J.J. (1985) Biochim. Biophys. Acta *829*, 387-396.