Biochimica et Biophysica Acta, 429 (1976) 1036—1040

BBA 67767

LACTATE RACEMASE

HYDROXYLAMINE-DEPENDENT ^{18}O EXCHANGE OF THE α-HYDROXYL OF LACTIC ACID

JAMES S. PEPPLE and DON DENNIS

Department of Chemistry, University of Delaware, Newark, Del. 19711 (U.S.A.)

(Received September 29th, 1975)

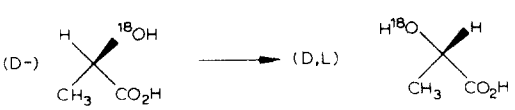
Summary

The lactic acid racemase (EC 5.1.2.1) derived from *Clostridium butylicum* catalyzes the racemization of the α-^{18}O label. The proposed α-carbonyl intermediate for the enzyme-catalyzed reaction has been previously shown to be trapped as an enzyme-bound oxime in the presence of hydroxylamine.

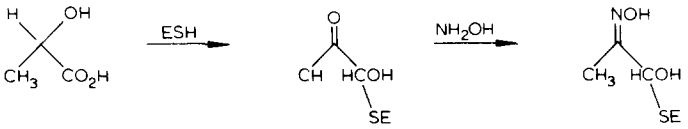
This report demonstrates that the formation of the inactive enzyme-bound oxime, followed by reactivation in the presence of an excess of competing free carbonyl (pyruvic acid) results in a complete loss of the α-^{18}O label from an original α-^{18}O-labeled lactic acid.

Introduction

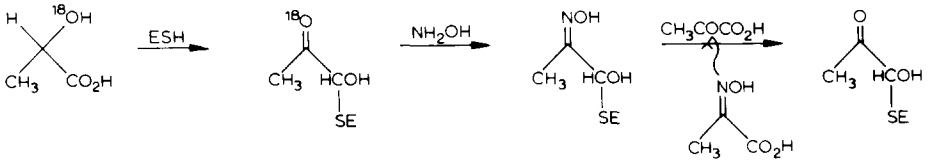
A series of studies in our laboratory has presented evidence in support of the hypothesis that a direct internal hydride shift is involved in the racemization of lactic acid as catalyzed by the enzyme lactate racemase (EC 5.1.2.1) derived from *Clostridium butylicum* [1—4]. This report describes the hydroxylamine-dependent α-^{18}O exchange of lactic acid during the course of the enzyme-catalyzed reaction. In the absence of hydroxylamine, α-^{18}O does not exchange out of lactic acid during the course of the racemization reaction [3].



In the presence of hydroxylamine an inactive enzyme-bound α-oxime of lactic acid derived from the proposed α-carbonyl intermediate has been characterized [4].



This report demonstrates that the formation of the inactive enzyme-bound oxime, followed by reactivation in the presence of an excess of competing free carbonyl (pyruvic acid) results in a complete loss of the α-^{18}O label from an original α-^{18}O-labeled lactic acid. Pyruvic acid itself does not exchange with lactate during the racemase reaction [1].



Experimental

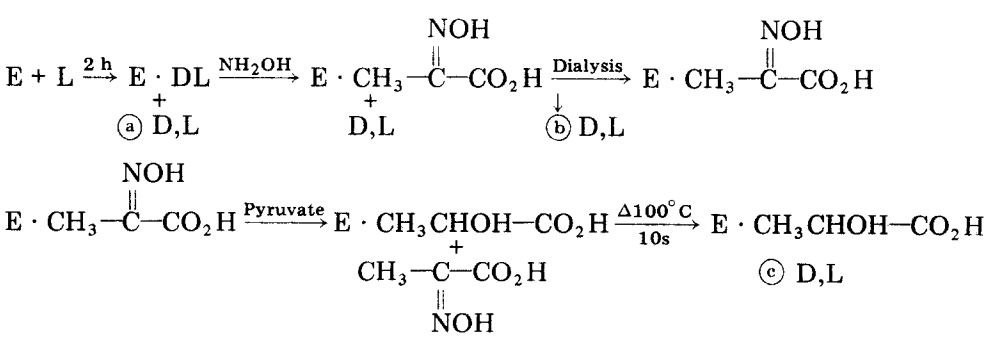
(α-^{18}O, 1-^{14}C)-labeled L(+)-lactic acid. The doubly labeled L(+)-lactic acid was made by the method of Shapiro and Dennis [3] except that [1-^{14}C]pyruvic acid replaced unlabeled pyruvic acid. The purified product was assayed enzymatically using beef heart lactate dehydrogenase, and then potentiometrically titrated with LiOH to yield the salt. The ^{18}O analysis was performed on neat samples of lactic acid introduced into a consolidated double focusing mass spectrometer and examined at *m/e* peaks of 45.061 and 47.061. A multiple mass ration run was made using a rapid repetitive alternation of the ion accelerating voltage. The enriched L(+)-lactate contained 4.7 atoms percent ^{18}O and had a specific radioactivity due to 1-^{14}C of $9.74 \cdot 10^4$ dpm/μmol.

Racemase enzyme preparation. Whole cells of *C. butylicum* were obtained from the New England Enzyme Center. The cells were grown under conditions described by Cantwell and Dennis [4]. The cells were suspended in 0.1 M sodium acetate buffer, pH 5.6, made 1 mM in DL-lithium lactate at a ratio of 1 : 4 (w/v) and sonicated for 3 min at 4° C. The suspension was centrifuged for 20 min at 15 000 rev./min to remove the cell debris. The supernatant was fractionated with $(NH_4)_2SO_4$ and the pellet collected from a 40—75% saturated solution was resuspended in a 0.1 M acetate buffer, pH 5.6, made 1 mM in DL-lithium lactate, and passed through a Sephadex G25 column. The active fraction was eluted with the void volume and contained 84% of the total protein. The specific activity was increased 6-fold in this step to the removal of a small molecular weight fraction. The preparation was very stable at this stage of purification. A second $(NH_4)_2SO_4$ precipitation step was then conducted primarily to concentrate the preparation to a small volume. The enzyme solution prepared from this pellet had a specific activity of 6 units/mg (1 unit = 1 μmol lactate racemized per 30 min at 37° C). The standard racemase assay was used as described by Cantwell and Dennis [4].

Standard procedure for isolation and purification of lactic acid from a reaction mixture. The reaction mixture was heated for 10 min at 100° C, cooled, ul-

trafiltered and evaporated to a small volume (5—10 ml), passed through a Dowex 50 cation-exchange resin (H^+ form), and the effluent lyophilized to an oily residue. A methanolic extraction was performed to precipitate nucleotides when they were present in the reaction mixture. An ethanolic extraction was also performed if hydroxylamine was present. The alcoholic solution was then taken to dryness, redissolved in 1.0 ml of water and placed on a Dowex 2 anion exchanger (formate form) and eluted with an exponential water/formic acid gradient over the range 10—100 mM. Tubes containing lactic acid (lactate dehydrogenase assay) were pooled and evaporated to dryness, extracted into anhydrous ether, filtered, evaporated to dryness and redissolved in water. Total lactic acid was determined by the titrimetric addition of LiOH.

Reaction mixture for NH_2OH trapping and α-^{18}O exchange. The reaction mixture contained a total of 8340 units of lactate racemase as a pellet obtained from a solution saturated to 75% with respect to $(NH_4)_2SO_4$, 400 μmol of (α-^{18}O, 1-^{14}C)-labeled L(+)-lactic acid and sufficient 0.1 M sodium acetate buffer, pH 5.6, to give a total volume of 310 ml. The reaction was incubated at 37° C for 2 h whereupon solid hydroxylamine was added to give a final concentration of 5 mM. The reaction mixture was then dialyzed for 25 h against 4 l of 5 mM hydroxylamine. The dialysate was changed every 5 h and monitored for the removal of unbound lactate by means of a radioactive assay for ^{14}C. The total dialysate was evaporated to a small volume to obtain a sample of the reaction mixture (b) for determination of the dilution factor due to the introduction of unlabeled lactate added initially from the enzyme preparation. The dialysed material was removed from the dialysis tubing, made 10 mM in pyruvate (2-fold molar excess of pyruvate/hydroxylamine), and maintained at 4° C for 24 h with constant stirring. The reaction was then heated at 100° C for 10 min to denature the enzyme and release the bound lactate. The lactate was isolated and purified according to the method described above.



Results and Discussion

After 120 min incubation of the reaction mixture, the percent racemization was determined by measuring the L(+)-lactate remaining and calculating the total lactate based on the ^{14}C specific activity of the L(+)-lactate initially introduced into the reaction. The extent of reaction was 50% using this method of

TABLE I

^{18}O CONTENT OF LACTATE DERIVED FROM VARIOUS INTERMEDIATES

(a) Racemase reaction assayed for L(+)-lactate (enzymatically), radioactive content of ^{14}C and ^{18}O content (mass spectrometer) at time "0" and 120 min. (b) Assay of the dialysis fluid equilibrated with the reaction mixture after the addition of NH_2OH to form the $E \cdot CH_3C(NOH)CO_2H$ adduct. These assays allowed the calculation of an appropriate dilution factor to compensate for the unlabeled lactate released from the original enzyme added. (c) Assays of the lactate derived from the $E \cdot CH_3C(NOH)CO_2H$ adduct after heat treatment of the pyruvate-reactivated complex. Details for these assays and the identity of the samples may be found in the text and a flow chart in the experimental section.

	Sample	L(+)-Lactate (μmol/ml)	[1-^{14}C] DL-Lactate (μmol/ml)	Atoms ^{18}O (%)	Dilution factor *	
					^{14}C	^{18}O
(a)	0 min	1.29	1.290	4.698	—	—
	120 min	0.90	1.201	—	—	—
(b)		2.26	3.00	3.9875	1.50	1.18
(c)		0.118	0.024	0.1865	9.76	—

* Assuming 100% racemization of enzyme-bound lactate.

calculation (Table I,a). A corrected value for the extent of reaction was made from the data obtained from the dialysate sample (Table I,b) where the ^{18}O dilution factor could be evaluated and employed to correct the percent racemization. The ^{18}O dilution factor obtained (4.698/3.9875 = 1.18) was employed to correct the total DL-lactate concentration (3.0 × 1.18 = 3.53) and allow the corrected calculation of percent racemization ([total lactate] − [L(+)lactate]/½ [total lactate] = 72.3%) at time 120 min. The dilution factor for calculating the atom percent ^{18}O for the enzyme-bound DL-lactate which exchanged with the original L(+)-lactate in solution was based on the [1-^{14}C]lactate content of the hydroxylamine trapped lactate (Table I,c). The [^{18}O, 1-^{14}C]lactate exchanged into the enzyme-bound lactate pool was calculated to have a dilution factor of 9.76 assuming that the enzyme-bound lactate was 100% racemized (D/L=1). The calculated atom percent ^{18}O for the enzyme-bound lactate was 4.698/9.76 = 0.481.

The observed atom percent ^{18}O content of 0.186, essentially natural abundance, (sample c) gave clear evidence that the complete loss of ^{18}O from the α-OH group had occurred when the inhibitor hydroxylamine was used to trap the α-carbonyl form of the enzyme-bound intermediate.

The mechanism of lactate racemization by the enzyme derived from *Clo. butylicum* has been shown to involve a transient carbonyl intermediate. The hydroxylamine-dependent α-^{18}O exchange data presented here constitute definitive proof that the carbonyl is in the α-position of the enzyme-bound lactic acid.

Acknowledgements

This investigation was supported in part by Research Grant GB-37309 from the National Science Foundation. Portions of the material were taken from a dissertation submitted to the University of Delaware (J.P.).

References

1 Dennis, D. and Kaplan, N.O. (1963) Biochem. Z. 338, 485—495
2 Shapiro, S.S. and Dennis, D. (1965) Biochemistry 4, 2283—2288
3 Shapiro, S.S. and Dennis, D. (1966) Biochem. Biophys. Res. Commun. 22, 635—639
4 Cantwell, A. and Dennis, D. (1974) Biochemistry 13, 287—291