Vol. 95, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1980 Pages 1205-1210

BACK EXCHANGE OF ^{18}O-LABELED AMINO ACIDS BY ERYTHROCYTES: THE POSSIBLE ROLE OF AMINO ACID TRANSPORT.

Keith L. Clay and Robert C. Murphy
Department of Pharmacology
University of Colorado School of Medicine
Denver, Colorado 80262

Received June 30,1980

SUMMARY

L-amino acids containing oxygen-18 at the carboxyl moiety have been found to undergo rapid loss of the stable isotope label through exchange with water in the presence of erythrocytes. This back exchange was found to be temperature dependent, stereospecific, and dependent on intact cells. The results suggest that an erythrocyte system catalyzes the formation of an intermediate which adds water to the labeled acid. This system has many characteristics similar to amino acid transport.

INTRODUCTION

The carboxyl oxygen atoms of amino acids are readily exchangeable with oxygen-18 water at elevated temperatures and low pH to yield stable isotopically labeled variants which are eminently suited as internal standards for quantitative analysis by gas chromatography-mass spectrometry (GC-MS) (1). The back exchange of ^{18}O-amino acids with water has been determined under various conditions of pH and temperature and it has been shown that ^{18}O-amino acids back exchange relatively slowly in either water or plasma. The fastest rate observed was for ^{18}O-leucine which back exchanged with a half-life of 32 days at room temperature at pH 1.5 (1). In plasma, ^{18}O-tyrosine back exchange was negligible, but when incubated with rat whole blood the isotope exchange occurred with a half-life of approximately two hours (2). The studies presented in this report extend those observations and offer a hypothesis for the basis of the biologically catalysed back exchange.

EXPERIMENTAL

^{18}O-H_2O of 99 atom percent excess ^{18}O (Prochem, Nutley, N.J.; Norsk Hydro, Oslo, Norway) acidified with HCl gas (Matheson, East Rutherford, N.J.) was used to prepare ^{18}O-amino acids as previously described (1). After incubation under the conditions described below, the amino acids were isolated by a procedure devised to minimize the possibility of back exchange during preparation (3). Isotopic composition analysis was carried out with a Finnigan 3200 GC-MS with mass spectral parameters and data acquisition under control of a Finnigan 6100 data system (Finnigan, Sunnyvale, Ca.) as previously described (1).

0006-291X/80/151205-06$01.00/0

Blood was taken from 300-500g male Sprague-Dawley rats by heart puncture after light ether anesthesia. Heparinized whole blood was then incubated with ^{18}O-amino acids. Washed erythrocytes were prepared by centrifugation twice in 5 volumes of physiological saline, phosphate buffered saline, Krebs-Ringer bicarbonate or Tyrode's incubation solution. There were not discernible differences with the use of the different incubation solutions. The packed erythrocytes were then re-suspended in an equal volume of buffer containing the desired ^{18}O-amino acid. Whole blood or erythrocyte suspensions were incubated in a shaking water bath and samples were taken for isotopic composition analysis at various intervals. When amino acid concentrations greater than 5 mM were used, osmotic compensation of the buffers was made by the procedures given by Christensen (4).

Intracellular measurements were made on aliquots of erythrocytes which were pipetted into a large volume of ice-cold buffer and centrifuged at 4°C. The pelleted cells were then re-suspended in ice-cold distilled water and kept at 4°C for one hour. After centrifugation, the amino acid was isolated from the lysate. Oxygen-18 content was determined by mass spectrometry as previously described (1).

RESULTS

Figure 1 illustrates the temperature dependence and the speed of the back exchange. Rat erythrocytes washed with a Krebs-Ringer bicarbonate buffer and then re-suspended in a buffer containing 200 µg/ml $^{18}O_2$-L-tyrosine were maintained at either 4° or 37°C and analysis of the amino acids in the extracellular fluid were carried out at intervals for 3 hours. The data presented if Fig. 1 are expressed as percent $^{18}O_2$ and are uncorrected for natural isotopic abundance. A decrease in atom percent excess ^{18}O could be the result of dilution with native amino acid or it could result from back exchange of oxygen-18 atoms with oxygen atoms of the aqueous milieu. In all of the data presented, therefore, isotopic analysis has included measurement of ions corresponding to $^{18}O_2$ amino acid, ^{18}O ^{16}O amino acid and $^{16}O_2$ amino acid. Back exchange involves a sequential replacement of ^{18}O atoms with ^{16}O atoms so that an increase in the ^{18}O ^{16}O species is observed and that increase is too large to be accounted for by natural isotopic abundance. In the case of dilution with $^{16}O_2$ amino acid, however, the abundance of the $^{16}O_2$ species increases with an increase of the ^{18}O ^{16}O species only in proportion to natural isotopic abundance. It is apparent that back exchange is quite rapid at 37°, but is negligible at 4°.

The back exchange is also quite stereospecific as illustrated in Figure 2. $^{18}O_2$-L-Leucine and $^{18}O_2$-D-leucine were incubated with washed rat erythrocytes at a concentration of 500 µg/ml (3.4 mM) and the isotopic species indicated were determined at intervals. Similar results have been obtained with a number of other L and D-stereoisomeric amino acids.

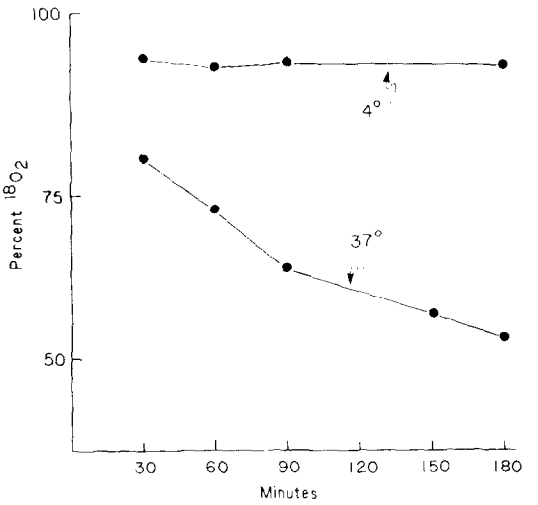

Figure 1 Back exchange of carboxyl oxygen atoms of $^{18}O_2$-L-tyrosine in the presence of washed rat erythrocytes.

The rate of back exchange of $^{18}O_2$-L-phenylalanine is linearly related to concentration with amino acid concentrations as high as 20 mM. Higher concentrations gave an indication of saturability, but limited availability of the isotopically labelled substrate precludes a definitive statement with regard to saturability at this time.

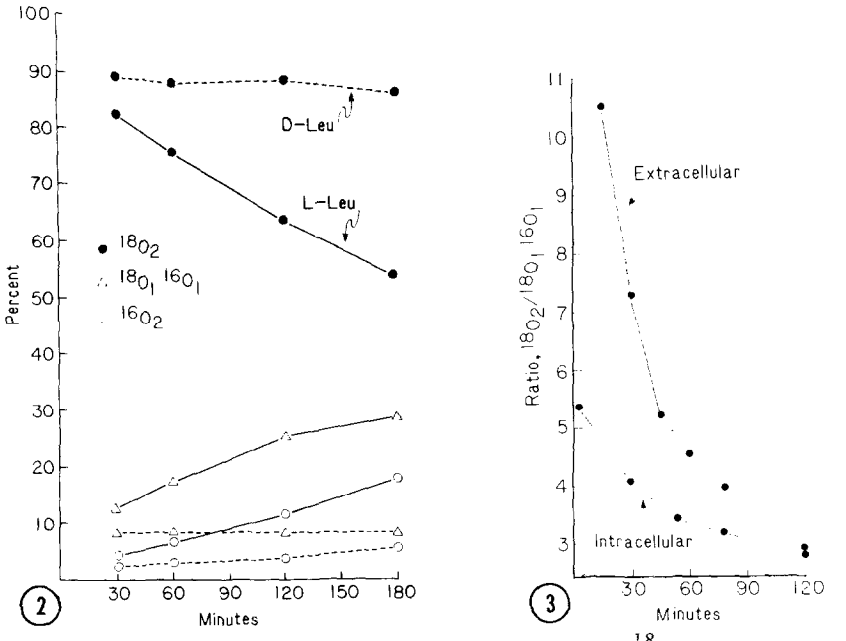

Figure 2 Back exchange of carboxyl oxygen atoms of $^{18}O_2$-L-leucine and $^{18}O_2$-D-leucine (3.4mM) in a suspension of washed rat erythrocytes.

Figure 3 Back exchange of $^{18}O_2$-L-tyrosine in a suspension of washed rat erythrocytes.

Because of the sequential nature of the back exchange, the ratio of the di-^{18}O species to the mono-^{18}O species is a sensitive measure of the back exchange if measurements are made at a time early enough that conversion of the ^{18}O ^{16}O species to the $^{16}O_2$ species does not introduce unacceptable error. Figure 3 illustrates that use of highly enriched $^{18}O_2$-L-tyrosine to estimate rates of back exchange in the intracellular and extracellular fluids of washed rat erythrocytes at relatively early times in the incubation. The rate of change of isotopic composition in the extracellular fluid is approximately twice the rate in the intracellular fluid.

Back exchange of ^{18}O carboxyl labelled amino acids has been observed with every amino acid used thus far, with one important exception. The only amino acid observed not to back exchange in the presence of erythrocytes has been $^{18}O_4$-L-aspartic acid.

Further experiments to characterize the back exchange phenomenon have included attempts to deplete erythrocytes of glucose by extensive washing and pre-incubation of the cells and attempts to replace sodium ions by washing and incubation in buffers with lithium ions replacing sodium ions. Neither of these maneuvers had any measurable effect on the rate of back exchange with $^{18}O_2$-L-tyrosine. Similarly, incubation of $^{18}O_2$-3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, the major metabolite of dopamine) with erythrocytes resulted in no measurable back exchange. The catalysis of back exchange, therefore, appears not to require Na^+ or glucose, and it is not a general reaction with all carboxylic acids.

Erythrocytes were disrupted by osmotic shock in distilled water and then ^{18}O-L-amino acids were incubated with the lysate and the membrane fractions. Neither fraction catalysed back exchange.

DISCUSSION

The temperature dependence (Fig. 1), stereospecificity (Fig. 2) and kinetics (Fig. 3) of the back exchange are all in qualitative agreement with known characteristics of erythrocyte amino acid transport (5). Direct comparison of rates obtained in these studies with literature values is difficult since other studies only report initial rates from experiments in which unidirectional flux is determined. These experiments were carried out under conditions in which a steady state was probably attained and in which it is

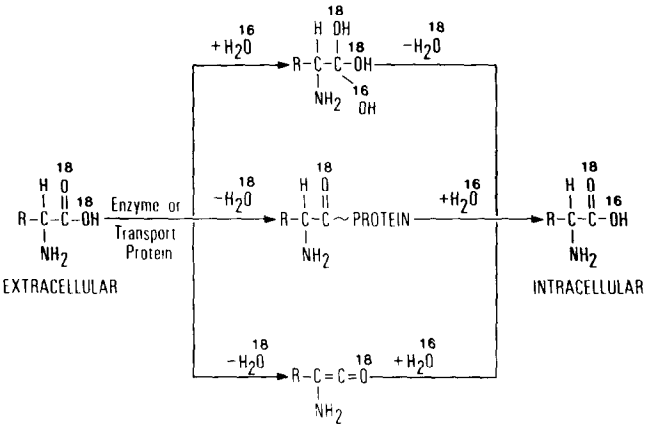

Figure 4 Possible intermediates in the biologically catalysed back exchange of carboxyl oxygen-18 labelled amino acids.

assumed that amino acid fluxes were equal in both transmembrane directions. The phenomenon being studied, therefore, would most closely correspond to the rapidly exchanging L system which is known to predominate in the red blood cells (6). The observation that analysis of extracellular fluid yielded rate change which was twice that observed when intracellular fluid was analyzed (Fig. 3) is consistent with an interpretation that the extracellular molecules have traversed the membrane twice as frequently as molecules in the intracellular fluid.

Aspartic acid is effectively excluded from erythrocyte transport (4, 5) and does not undergo back exchange. If the catalytic event were simply a membrane-bound system on the exterior surface of the cell, it is difficult to understand why there was no back exchange with this amino acid. Similarly, the experiments with lysate from osmotically shocked erythrocytes would indicate that an enzyme of the cytoplasm cannot by itself catalyse oxygen exchange of amino acid carboxyl oxygens. Neither membrane nor cytoplasmic fractions of osmotically disrupted erythrocytes catalysed back exchange of ^{18}O amino acids, which indicates that some function of intact cells is necessary for catalysis of the back exchange.

The results of the oxygen isotope exchange experiments have been consistent with a mechanism which is rate-limited by transport of the oxygen-18 amino acid. We would like to forward the hypothesis that the mechanism of amino acid transport in erythrocytes involves formation of some intermediate species which leads to carboxyl oxygen exchange.

Figure 4 illustrates three possible intermediate species whose formation during amino acid transport could explain the data thus far obtained. Each of the postulated intermediates involves reversible addition and removal of one molecule of water during the process of membrane transport. Use of stable isotope labeled amino acids and an appropriate biological system will allow discrimination between these three possibilities since each model predicts different isotopic distribution in the transported molecule.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Institutes of Health, AA-03527 and NS-09199 and a Career Development Award HD 00128 to RCM.

REFERENCES

1) Murphy, R.C. and Clay, K.L. (1979). Biomed. Mass Spectrom. 6: 309-314.

2) Murphy, R.C., Anderson, F.A. and Clay, K.L. (1979). in Proceedings of the Third International Conference on Stable Isotopes, pp. 139-146. Academic Press, New York.

3) Clay, K.L. and Murphy, R.C. (1979). J. Chromatogr. 164: 417-426.

4) Christensen, H.N. (1975). Biological Transport 2nd Ed., pp. 456-459, W.A. Benjamin, Massachusetts.

5) Young, J.D. and Ellory, J.C. (1977) in Membrane Transport in Red Cells, pp. 301-325. Academic Press, New York.

6) Christensen, H.N. (1973). Fed. Proc. 32: 19-28.