Calculation of the rate constant of a reversible reaction from the chromatographic peaks for muramyldipeptide anomers

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The calculation of the rate constants of a reversible reaction in both directions involves the separation of one of the components and measurement of the velocity of its conversion to the other component of the equilibrium. This presents no problem under conditions such as those in crystallization, where the reaction does not proceed or if the extent of the reaction during the time needed for the separation can be neglected. However, it may become difficult in situations where the progress of the reaction competes even with a fast separation process such as high-performance liquid chromatography (HPLC).*

We encountered such a case during the chromatographic separation of alpha and beta anomers of muramyldipeptide I. At a particular elution velocity we observed a plateau between the peaks of the alpha and beta anomers (Fig. 1), which increased when the elution time was prolonged. This effect is consistent with the interpretation that the plateau is composed of alpha and beta anomers generated from the beta and alpha anomer peaks during the chromatography. This interpretation is in accord with that for the chromatographic separation studied by Melander et al.1 for the cis–trans interconversion of proline dipeptides. They dealt with an analogous splitting of chromatographic peaks. We have derived an equation for the calculation of the velocity constants of an equilibrium reaction that is first order in both directions, from the chromatographic peaks. A knowledge of the equilibrium constant, equal to the ratio of the areas of the peaks of the anomers at an infinitely high elution rate, is necessary. It

* Generation of a product during chromatographic separation is commonplace with separations of radioisotopes with short half-lives, which is, of course, a simple first-order process.
Fig. 1. Chromatographic separation of anomers of I at a flow-rate of 0.3 ml/min. Areas (in arbitrary units): A, 89.2; B, 50.1; C, 30.4

Fig. 2. Chromatographic separation of anomers of I at a flow-rate of 6.8 ml/min.
can be obtained from a chromatographic experiment in which the elution velocity is set high enough to render the plateau below the detection limit (Fig. 2).

EXPERIMENTAL

The muramyldipeptide I (MDP) was prepared in our Institute. The chromatographic separations were performed on an SP-8700 chromatograph equipped with an SP-8400 UV detector and an SP-4100 integrator (Spectra Physics, Santa Clara, CA, U.S.A.). A 25 × 0.4 cm I.D. Zorbax ODS column (DuPont, Wilmington, DE, U.S.A.) with 0.1% trifluoroacetic acid with 1% methanol at 34°C as the mobile phase was used. For the determination of the velocity constant with an isolated anomer, 0.5 mg of I was separated at a flow-rate of 1.5 ml/min. The time of elution of the anomer peak that was collected was taken as zero. The anomer solution was kept at 34°C and samples were taken at several time intervals and analysed at a flow-rate of 6.8 ml/min. The equilibrium constant of the reaction was determined at the same flow-rate with a sample of I dissolved in the mobile phase equilibrated at 34°C for 2 days.

The velocity constant was calculated from the equation derived for an equilibrium reaction first order in both directions:

\[ k = \frac{K}{(t_x \cdot (1 + K)) \cdot \ln[Kc_0^A/(Kc_0^A - (1 + K) c^C)]} \]

where \( c_0^A \) is the starting concentration of A and \( c_x^A \) is the concentration of A at time \( t_x \), \( K = 1.79 \). A mean value from four time readings was \( k = (3.45 \pm 0.4) \cdot 10^{-2} \) min\(^{-1}\). Starting values for the calculation of velocity constants according to eqn. 6 (see Results and Discussion) were obtained by graphical integration of the peak areas shown in Fig. 1.

RESULTS AND DISCUSSION

Separation of anomers was observed during chromatography of peptidoglycans. At a sufficiently low flow-rate a plateau appeared between the peaks, the height of which increased with an increase in temperature of the mobile phase. A similar plateau was also found on chromatographic records of sugars and proline peptide separations. Apparently, the plateau consists of a mixture of anomers (conformers in the case of proline peptides) generated during the chromatographic separation. Therefore, its area should be related to the velocity of their interconversion, in the particular case of an equilibrium that is established by first-order reactions in both directions. Let

\[ A \xrightleftharpoons[k_1]{k_2} B \quad (1) \]

be the equilibrium reaction between the anomers; anomerization is known to be essentially first order in both directions.

The elution pattern (Fig. 1) consists of peaks A* and B* of A and B and a plateau C* which consists of both A and B produced from the starting B_0 and A_0 during the
elution. Because of the continuous separation of products from starting compounds, only the remainder of the starting compound takes part in the velocity expression. Therefore, we can write for the velocity constant of one of the directions

\[ k_2 = \frac{1}{t_B} \cdot \ln \left( \frac{B_0}{B_0 - x} \right) = \ln \frac{1}{t_B} \cdot \left( \frac{B_0}{B^*} \right) \]  

The starting concentration \( B_0 \) is available as a sum with \( A_0 \), because at any time \( t \)

\[ A_0 + B_0 = A^* + B^* + C^* \]  

Using the equilibrium constant, obtained in a separate experiment:

\[ K_{eq} = \frac{A_0}{B_0}; A_0 = K_{eq}B_0 \]  

and combining with eqn. 3, we obtain

\[ B_0(1 + K_{eq}) = A^* + B^* + C^*; B_0 = \frac{A^* + B^* + C^*}{1 + K_{eq}} \]  

and therefore

\[ k_2 = \frac{1}{t_B} \cdot \ln \left[ \frac{A^* + B^* + C^*}{B^* (1 + K_{eq})} \right] \]  

and

\[ k_1 = \frac{k_2}{K_{eq}} \]

The rate constant of beta to alpha anomer conversion \( (k_2) \) calculated according to eqn. 6 was found to be \( 3.25 \cdot 10^{-2} \text{ min}^{-1} \) (the error of the calculation depends mainly on the integration of the areas \( A^*, B^* \) and \( C^* \); the error was found to be \( \pm 22\% \) based on five determinations of the areas). For comparison, the same constant calculated from data furnished by several chromatographic experiments with the isolated alpha and beta anomers was found to be \( (3.45 \pm 0.4) \cdot 10^{-2} \text{ min}^{-1} \), both at 34°C. The fair agreement corresponds roughly to the precision of the input data. It also means that the velocities of the studied reactions are not significantly influenced by the process of the chromatographic separation itself (i.e., absorption and desorption on the immobilized non-polar phase)\(^6\). A clear advantage of our approach is the need for only two chromatographic experiments for the determination and no strict requirements on the purity of the studied compounds (cf., Figs. 1 and 2). The observations of chromatographic patterns with the characteristic plateau such as that described here can be found in the literature\(^1,7,8\). In a number of such instances this
method would allow the calculation of the velocity constants of the equilibria involved. However, it is limited to the reaction conditions given by the process of chromatographic separation.

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REFERENCES

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