DETERMINATION OF A FIRST-ORDER RATE CONSTANT BY DIFFERENCE GEL CHROMATOGRAPHY: DISSOCIATION OF INSULIN BY ALKALI

S.J. LOVELL*, L.W. NICHOL† and D.J. WINZOR*

*Department of Biochemistry, University of Queensland, St. Lucia, Queensland 4067, Australia**

and

Department of Physical Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. 2601, Australia†

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1. Introduction

An earlier investigation [1] has shown that quite unusual elution patterns might be obtained in a difference gel chromatography experiment involving the transfer of a solute from one solvent system in which it is stable to one in which it undergoes time-dependent isomerization. However, no such patterns have been observed experimentally, and thus the potential for evaluating a rate constant by gel chromatography remains untested. The aim of the present communication is to explore this aspect using the recently reported alkaline dissociation of insulin from dimer to monomer [2]. The required theory is based on a set of partial differential equations expressing mass conservation during migration: this set, together with the relevant boundary conditions, is given in eq. 1 of ref. [1]. Although the set was formulated originally for isomerization ($A \rightarrow B$), it also describes the irreversible dissociation reaction $A \xrightarrow{k} nB$, where k denotes the first-order rate constant, and hence the solutions of these differential equations (i.e. descriptions of elution profiles) are also pertinent to the dissociating system. The value of 0.011_3 min^{-1} that is obtained for k by this method is in accord with zonal studies of the insulin dissociation.

Address for Correspondence: Professor L.W. Nichol, Department of Physical Biochemistry, The John Curtin School of Medical Research, Australian National University, P.O. Box 334, Canberra City, A.C.T. 2601, Australia.

2. Experimental

A concentrated solution (20 mg/ml) of crystalline bovine zinc insulin (Sigma) was adjusted to pH 11 with NaOH and the resulting solutions (2–3 ml) applied to a 2.1 × 14 cm column of Sephadex G-25 equilibrated with 0.008 M NaOH. Eluate fractions (2 ml) were analyzed spectrophotometrically at 280 nm and the contents of the tubes corresponding to the protein peak pooled. This procedure proved to be necessary in order to rid the insulin sample of contaminants that greatly affected its gel chromatographic behaviour on Sephadex G-75.

The stock solution of insulin in 0.008 M NaOH was then diluted to a concentration of 0.6 mg/ml and subjected to either the frontal [3] or difference [4] technique of gel chromatography. In the latter experiments 80 ml of insulin in 0.008 M NaOH was applied to a 1.4 × 34.5 cm column of Sephadex G-75 equilibrated with 0.05 M NaOH and thermostated at 25°C. A flow rate of either 7.4 or 38.4 ml/hr was maintained by means of a peristaltic pump. The effluent from the column was divided into 1 ml fractions which were diluted with an equal volume of 2 M NaOH prior to absorbance measurements at 290 nm. Alkaline pretreatment was required to achieve identical extinction coefficients for the protein in the two environments.

In a second type of kinetic experiment, insulin (1 ml, 4.5 mg/ml) was made 0.05 M with respect to NaOH and incubated at 25°C for specified time inter-

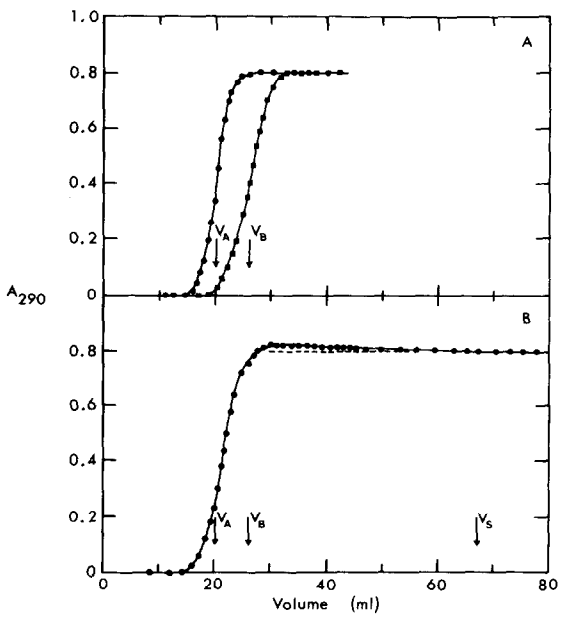

Fig. 1. Elution profiles obtained in Sephadex G-75 chromatography of bovine zinc insulin in the following solvent media: A, 0.008 M NaOH (•) and 0.05 M NaOH (▪), the sample being incubated for 10 hours prior to application; B, insulin in 0.008 M NaOH applied to a column pre-equilibrated with 0.05 M NaOH. In each case a flow rate of 38.4 ml/hr was maintained through the 1.4 × 34.5 cm column of Sephadex G-75.

vals, after which HCl was added to decrease the molarity of NaOH to 0.008 M. The samples were then analyzed by zonal chromatography on the 1.4 × 34.5 column of G-75, equilibrated in this instance with 0.008 M NaOH. Control experiments established that in this medium neither dissociation of dimeric insulin nor association of monomer occurred to any detectable extent during a 5 hour incubation period.

3. Results

Advancing elution profiles for insulin incubated for 10 hr in 0.008 M and 0.05 M NaOH, are shown by circles and squares, respectively, in fig. 1A, which confirm the earlier observation [2] that insulin has a larger elution volume in the more alkaline environment: the change in elution volume from 20.0 ml (V_A) for protein in 0.008 M NaOH to 26.0 ml (V_B) in 0.05 M NaOH has been attributed to a dimer → monomer interconversion [2]. Fig. 1B presents the results of a difference gel chromatography experiment conducted at a flow rate of 38.4 ml/hr in which insulin in 0.008 M NaOH was loaded onto a column pre-equilibrated with 0.05 M NaOH. At effluent volumes greater than the elution volume of the solvent front (V_S = 67.0 ml) the protein migrates down the column in its original environment (0.008 M NaOH) and maintains a plateau of original concentration $c^{-\alpha}$ = 0.6 mg/ml. At volumes less than V_S protein has migrated into the more alkaline environment and the consequences of the kinetically controlled dissociation are evident. Thus the concentration is not maintianed at the plateau value ($c^{-\alpha}$) but increases steadily with decreasing volume until it reaches a maximum value at an elution volume approximating to V_B. With further decrease in effluent volume the concentration decreases rapidly to zero in the vicinity of V_A. These features parallel closely those illustrated in fig. 1B of ref. [1] when it is realized that the latter theoretical pattern takes no account of diffusional spreading.

Meggitt et al. [1] have suggested use of the following analytical integral to evaluate k.

$$Q = [\dot{V}(V_B - V_A)(V_S - V_A)c^{-\alpha}/kV_A(V_S - V_B)] \times \{1 - \exp[-kV_A(V_S - V_B)/\dot{V}(V_S - V_A)]\} \quad (1)$$

In this expression $\dot{V}$ denotes the volume rate of flow of the column, and Q the amount of solute eluted between V_A and the maximum in concentration occurring at V_B. They noted that the experimental measurement of Q should include the small amount of material diffusing ahead of V_A, but did not comment on the possibility that the experimentally observed maximum might be displaced slightly from V_B (the upper limit of integration), also as a consequence of diffusion. Accordingly, three estimates of Q were obtained by trapezoidal integration using as the upper limits volumes corresponding to V_B, the maximum, and the mean of those values. This permitted the evaluation of k from eq. 1 with the aid of a digital computer: the mean value of k obtained was $0.011_3 \pm 0.001_3$ min^{-1}, the extreme values being obtained with V_B and the maximum as the upper limit.

A similar result was obtained in an experiment of identical design but employing a flow-rate of 7.4 ml/hr, the profile being shown in fig. 2: the solid line in this case denotes the theoretical pattern calculated from

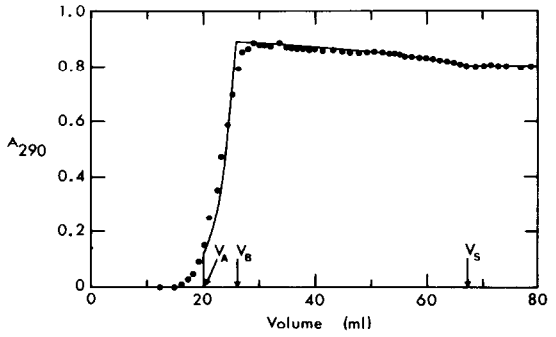

Fig. 2. Elution profile obtained in a difference gel chromatography experiment under conditions identical with those for fig. 1B except that the flow rate was decreased to 7.4 ml/hr. The solid line denotes the theoretical profile calculated ignoring effects of diffusional spreading.

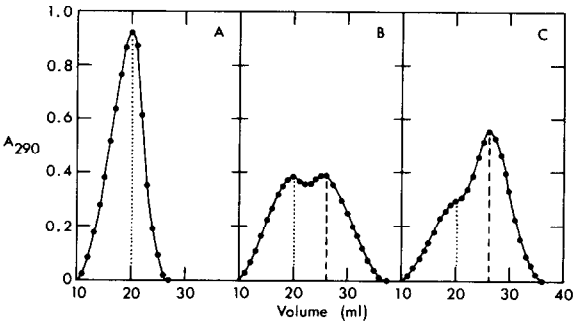

Fig. 3. Zonal elution profiles obtained in Sephadex G-75 chromatography of insulin solutions incubated in 0.05 M NaOH for different periods of time: A 0 min; B 60 min; C 120 min. Dotted and dashed lines denote V_A and V_B, respectively. The column of Sephadex G-75 (1.4 × 34.5 cm) was equilibrated with 0.008 M NaOH and operated at a flow rate of 38.4 ml/hr.

eq. 11 and 12 of ref. [1] with the mean value of k. The agreement is reasonable when note is again taken of diffusional spreading effects. A major difference between the experimental profiles in figs. 1B and 2 is the increased value of the maximum concentration in the experiment with slower flow rate, a finding also in accord with theoretical prediction [1].

The value of k corresponds to a half life ($t_{1/2}$) of 61 ± 7 min, whereas an estimate of about half this magnitude would have been inferred from zonal gel chromatography studies (fig. 2 of ref. [2]). Because of the sensitivity of insulin association to variation in zinc content [5], similar zonal studies have been performed with the present preparation of insulin, the results being shown in fig. 3. From these elution profiles it is clear that a 50:50 distribution of monomer and dimer is obtained after incubation in 0.05 M NaOH for approximately 60 min, a finding in basic agreement with that obtained by difference gel chromatography.

4. Discussion

In general the determination of $t_{1/2}$ and hence k from zonal analysis requires a graphical resolution of incompletely resolved peaks. Although this disadvantage may in principle be overcome by evaluating the time-dependence of the median bisector of the observed biomodal zones, it is implicit in the zonal method that conditions can be found under which the dissociation reaction may be frozen. With the present system control experiments have verified that this is possible. The difference gel chromatographic procedure suffers from neither disadvantage inasmuch as graphical resolution is not required, and there is no need to search for a medium that permits analysis of the relative proportions of species at intermediate states as the system proceeds irreversibly with time from an initial to a final state. It is therefore hoped that the present illustration of the evaluation of a rate constant by difference gel chromatography may prove useful in elucidating other kinetically controlled interconversions.

Acknowledgements

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