PN 10032

A time-dependent effect of K^+ on L-threonine dehydrogenase

Following the demonstration by ELLIOTT[1-3] of the conversion of threonine to aminoacetone by washed cell suspensions of *Staphylococcus aureus*, NEUBERGER AND TAIT[4,5] described an enzyme present in cell-free extracts of *Rhodopseudomonas spheroides* which catalyses the reaction:

$$\text{L-threonine} + \text{NAD}^+ \rightarrow \text{aminoacetone} + \text{NADH} + \text{H}^+ + \text{CO}_2$$

A similar enzyme was shown to be present in cell-free extracts of *S. aureus*[6].

The present communication describes an unusual type of activation of this enzyme by K^+. (The term 'activation', throughout this communication, should be taken to mean 'apparent activation'. It may be, in fact, true activation, or stabilisation, or both, as these different effects cannot be distinguished at present.)

The enzyme extract was prepared by sonic disruption of *S. aureus* cells suspended in 0.1 M potassium phosphate (pH 7.0), containing 0.2 M KCl. The sonicate was centrifuged at 105 000 × *g* for 1 h and the supernatant, which contained the enzyme, was stored in the frozen state until required.

The assay mixture contained, in a total volume of 3.0 ml, 100 μmoles of Tris–HCl buffer (pH 8.5), 300 μmoles of L-threonine, 1 μmole of NAD^+, an NAD^+-regenerating system consisting of 2 μg of crystalline lactate dehydrogenase (EC 1.1.1.27) and 30 μmoles of potassium pyruvate, and 0.75 ml of bacterial extract (about 1.7 mg of protein). In addition, sufficient KCl was added so that the final concentration of K^+ was 0.3 M. The composition of the reaction mixture was thus independent of the KCl content of the enzyme solution. Incubation were carried out in 25-ml

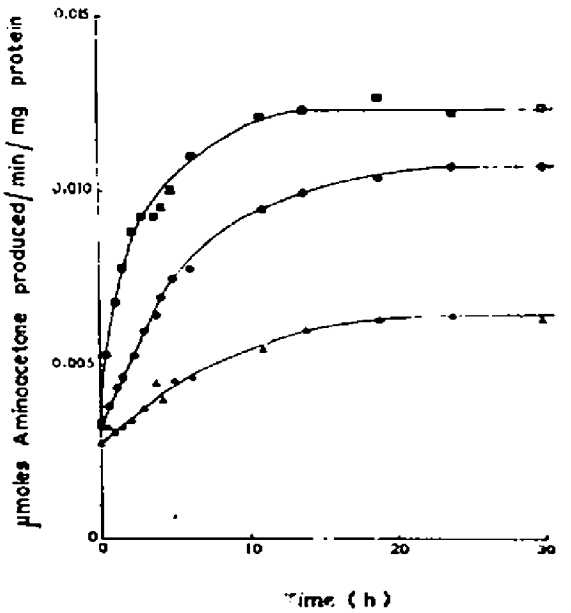

Fig. 1. Time curve for the reactivation of dialysed enzyme by KCl (■—■, 1.00 M; ●—●, 0.30 M; ▲—▲, 0.05 M). The enzyme solutions were incubated at 0°, and the activities were assayed, after various times, as described in the text.

erlenmeyer flasks, which were shaken aerobically at 37°. After 15 min the reaction was stopped by the addition of 0.6 ml of 25% $HClO_4$. After removal of the precipitated protein and $KClO_4$ by centrifugation, a sample of the supernatant, neutralised by NaOH, was taken for the determination of aminoacetone by the method of MAUZERALL AND GRANICK[7].

When the enzyme was dialysed against 100 vol. of water at 4° for 12 h, it was found to lose most of its initial activity. However, if, subsequent to dialysis, the enzyme was incubated with KCl at 0° and then assayed, some of the original activity was recovered; this preliminary incubation was needed even though KCl was routinely present in the assay mixture.

As might be expected from these observations, the reactivation of the dialysed enzyme by KCl is very slow (Fig. 1); at 0°, up to 24 h incubation is required to obtain maximal restoration of activity. Both the rate and degree of reactivation increase as the concentration of KCl, with which the dialysed enzyme is incubated, is raised. Fig. 2 shows the extent of reactivation at different concentrations of

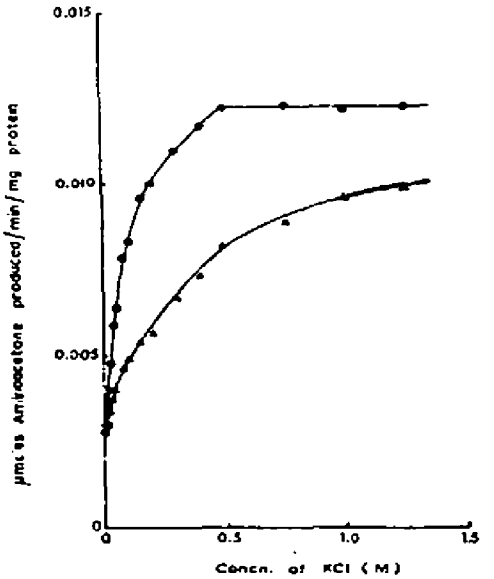

Fig. 2. Effect of concentration on the extent of reactivation of the dialysed enzyme, after incubation for 3.7 h (▲—▲) and 28.8 h (●- -●). The conditions were as described for Fig. 1.

KCl, after incubation for both 3.7 h and 28.8 h. Incubation with 0.5 M KCl for at least 24 h was sufficient for maximal restoration of enzymic activity.

In order to eliminate the possibility that divalent-metal-ion contaminants in the KCl were responsible for the reactivation, incubation with KCl was carried out in the presence of 1 μM–10 mM EDTA (pH 7.0). This caused no alteration in either the rate or extent of reactivation.

Specificity studies show that it is the cation, rather than the anion, which is effective in causing reactivation of the dialysed enzyme. K^+ can be replaced by NH_4^+, Rb^+ and Cs^+; Li^+ and Na^+ are almost inactive.

Dialysed enzyme, which has not been incubated with KCl, still shows appreciable activity (about 30% of the maximum attainable) which may be due to the residual amount of potassium in the enzyme solution, particularly as determination, by flame photometry, showed a concentration corresponding to 3 mM K^+. Therefore, it is impossible, at present, to decide whether the potassium-free enzyme has any residual activity.

A straight line is obtained for the LINEWEAVER–BURK[8] plot of enzymic activity after equilibration with different concentrations of KCl (Fig. 3), if it is assumed

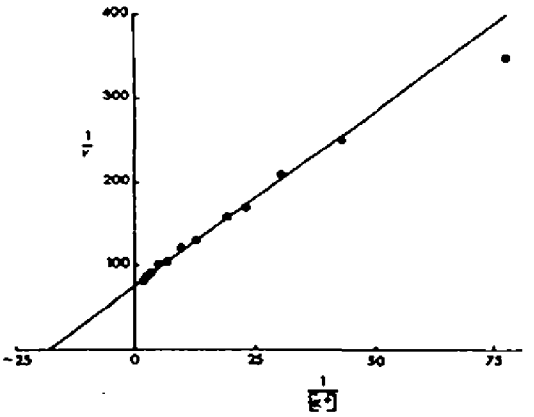

Fig. 3. LINEWEAVER–BURK[8] plot of the enzymic activity at different concentrations of KCl. The results were those used for the upper curve of Fig. 2, except that they were corrected for the residual potassium content of the dialysed enzyme. Values up to 0.5 M KCl are plotted.

that the 3 mM K^+, present in the dialysed enzyme, is entirely responsible for its residual activity. This suggests that each active centre combines with only one K^+. A value of 0.055 M was obtained for the K_m of K^+. It seems possible, therefore, that the active form of the enzyme is a potassium–apoenzyme complex formed by very slow combination of K^+ with inactive apoenzyme. The results would also be explained by the general schemes put forward by MALMSTRÖM AND WESTLEY[9] and RABIN[10], in which the reaction of an enzyme with a metal ion is instantaneous, the slow reaction being the interconversion of two forms of the enzyme. It is also possible that the slow combination of K^+ is concerned with stabilisation of the enzyme rather than activation, as was found by ROSENBERG[11] for aminoacyl-L-histidine hydrolase (EC 3.4.3.3). In any case, the high concentrations of KCl required presumably reflect the low stability constant of the potassium–apoenzyme complex.

ATP:D-fructose 6-phosphotransferase (EC 2.7.1.4)[13], L-threonine hydro-lyase (deaminating) (EC 4.2.1.16)[14] and tryptophan hydrolase[15], have been shown to be activated by K^+. However, in all these cases the activation is apparently instantaneous. The only other cases of slow activation of enzymes by metal ions which have been reported are the activation of certain peptidases[16] and L-arginine ureohydrolase (EC 3.5.3.1)[17] by Mn^{2+}. In the case of leucine aminopeptidase (EC 3.4.1.1) the time required for activation decreases with purification of the enzyme[18].

I wish to thank Dr. W. H. ELLIOTT for his interest and advice, Professor A. G. OGSTON for helpful discussions, Mr. J. LEE for skilled technical assistance, and the Australian National University for a research scholarship, during the tenure of which this work was carrried out.

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Received December 3rd, 1962
Revised manuscript received January 24th, 1963

Biochim. Biophys. Acta, 67 (1963) 682–685

PN 10031

Free radical in Michaelis complex of D-amino acid oxidase

In the course of our study on the mechanism of enzyme action, we succeeded in crystallizing the Michaelis complex of D-amino acid oxidase (D-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.3) under anaerobic condition[1]. Both the faint purple-coloured crystal and the purple-coloured mother liquid showed the same absorption band at 500–600 mμ. On aeration, the crystal was converted into an equimolar amount of the holoenzyme and pyruvate accompanying by the formation of H_2O_2. These findings led us to suppose that the complex consisted of the semiquinoid form of the holoenzyme and the activated substrate. To test for the possible presence of free radicals in the complex, an electron-spin-resonance (ESR) study has been carried out on both the crystal and the mother liquid.

The ESR spectra were measured using a X-band EPR spectrometer Varian type V 4500 with 100-kcycles field modulation. The measurements were made in the same way as KUBO *et al.*[2]. For the measurements, a capillary type cell (Fig. 1) was devised and made from quartz glass.