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TIME-DEPENDENT ACTIVATION AND INACTIVATION OF PIG BRAIN GLUTAMINASE

GILLIAN A. NIMMO* and KEITH F. TIPTON†
Department of Biochemistry, Tennis Court Road, Cambridge CB2 1QW, U.K.

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Abstract—Time-dependent changes in the activity of soluble and membrane-bound glutaminase from pig brain were examined. The membrane-bound enzyme was relatively stable to inactivation as a consequence of dilution and did not show time-dependent activation by phosphate or borate ions. In contrast the soluble enzyme lost activity rapidly when diluted but the presence of glutamine protected against this inactivation and the competitive inhibitor albizziin also afforded some protection. Inactivation occurred even more rapidly in the presence of glutamate. The stability of the soluble enzyme depended on the buffer in which it was stored and storage in Tris-HCl buffer pH 7.4 resulted in a loss of its ability to be activated by phosphate and borate ions. The kinetics of the activation of the soluble enzyme in Tris-HCl buffer by borate and phosphate ions showed the order of the reaction with respect to enzyme concentration to be 3.5 and 1.9 respectively with Hill constants of 1.8 for borate and 2.2 for phosphate. Activation of a preparation of the soluble enzyme stored in a borate-containing buffer by phosphate ions gave an order of 1.7 with respect to enzyme concentration and a Hill constant for phosphate of 2.4. These results were consistent with the activation processes involving aggregation of the enzyme and the smaller form of the enzyme in Tris-HCl buffer having at least two binding sites each for phosphate and borate ions. The implications of these time-dependent phenomena for assay of the activity of the soluble enzyme are also discussed.

The enzyme glutaminase (E.C.3.5.1.2) catalyses the hydrolysis of L-glutamine to form L-glutamate and ammonium ions. Pig brain mitochondria have been shown to contain two major forms of glutaminase, a readily extractable soluble form and a membrane-bound enzyme [1]. Both of these forms were activated by phosphate. The soluble enzyme has been purified to homogeneity from pig brain [2] and it appears to be the same as the glutaminase purified from the same source by Svenneby *et al.* [3], since both enzymes become aggregated in buffers containing phosphate and borate, a process which is reversed on removal of these ions. Glutaminases with similar properties have been prepared from pig kidney [4], rat kidney [5], ox brain [6], and several rat tissues [7]. The rat kidney enzyme has been reported to be immunologically identical to that from rat brain [8] although structural differences between the enzymes from pig kidney and brain have been observed [3, 9].

The aggregation that occurs in phosphate-borate buffer is accompanied by an increase in the specific activity of the enzyme and it is a relatively slow process [10]. This slow activation upon the addition of phosphate-borate has been studied for the pig brain enzyme [11] and the pig kidney enzyme [10, 12]. Membrane bound glutaminase was not activated in this time-dependent manner by preincubation in phosphate-borate [1].

The physiological relevance of these slow changes in the properties of soluble glutaminase, or hysteretic effects [13], is not clear. They must, however, be taken into account when assaying the enzyme if meaningful and reproducible results are to be obtained which can be compared with those of other workers and failure to do this may account for some of the discrepancies between previous reports. In this paper we report the results of kinetic studies of these activation and inactivation reactions and comment on the conditions necessary to minimize these effects.

MATERIALS

Ox liver glutamate dehydrogenase (E.C.1.4.1.3), NADH, NAD and 2-oxoglutarate were obtained from C. F. Boehringer und Soehne (Mannheim GmbH, F.D.R.) Sephadex G-25 was obtained from Pharmacia (Uppsala, Sweden). L-2-amino 3-ureido propionic acid (albizziin) was obtained from Koch-Light Laboratories (Colnbrook, U.K.), and was recrystallised twice from aqueous ethanol. L-glutamine was obtained from Sigma (London, U.K.). L-Glutamic acid was obtained from Serva GmbH and Co. (Heidelberg, F.D.R.). Dithiothreitol (DTT), Analar Tris and the other chemicals used in the work were obtained from BDH Ltd. (Poole, U.K.)

Membrane-bound glutaminase was prepared from pig brain mitochondria by the method described previously [1]. Soluble glutaminase was purified to apparent homogeneity from pig brain as described previously [2].

* Author to whome correspondence should be addressed at: Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

† Present address: Department of Biochemistry, Trinity College, Dublin 2, Ireland.

METHODS

Glutaminase assays

Glutaminase activity was determined at 30° using one of the following three methods. All of the assays described in this paper contained ≤1 μg/ml of soluble glutaminase. One unit (U) of enzyme activity is defined as the amount catalysing the formation of 1 μmol of product in 1 min.

(a) *Coupled assay for ammonium ions.* This assay was similar to that described by Kvamme *et al.* [4]. The assay mixture contained in a total volume of 1 ml, 100 mM triethanolamine hydrochloride-NaOH buffer, pH 7.4, 0.1 mM EDTA, 3 mM 2-oxoglutarate, 0.1 mM NADH, 10 U glutamate dehydrogenase (0.5 mg protein under these conditions in 50 per cent glycerol), 5 mM glutamine and enzyme sample. The oxidation of NADH was monitored at 340 nm as described previously [1].

(b) *Assay for ammonium ions using an ion-selective glass electrode.* The assay mixture contained, in a total volume of 10 ml, 100 mm Tris, 0.1 mM EDTA adjusted to pH 7.4 with HCl, 5 mM glutamine and enzyme sample. The rate of production of NH_4^+ ions was measured using an EIL ion-selective glass electrode (Electronic Instruments Ltd., Richmond, U.K. Type GKN 33) coupled via a pH meter to a strip-chart recorder as described previously [1].

(c) *Coupled assay for glutamate.* This was a modification of the stopped assay for glutamate of Bernt and Bergmeyer [14]. The standard assay mixture contained, in a total volume of 1 ml, 0.5 M glycine-hydrazine hydrate buffer pH 9.0 (freshly prepared each week), 2 mg NAD^+, 5 mM glutamine, 3–3.5 U glutamate dehydrogenase (0.5 mg protein under these conditions in 50 per cent glycerol) and enzyme sample. The reduction of NAD^+ was monitored at 340 nm. Under these conditions the assay gave 99 per cent of the true rate of reaction after not more than 3 min with the enzyme activities used, as determined experimentally and also as calculated by the method of McClure [15]. The blank rate in the absence of glutaminase was fairly high (0.01–0.02 absorbance units/min). Glutaminase activity was found to be lower in this buffer than in those used in the other assays.

Calculation of the order of the activation reactions

Time courses of the activation of soluble glutaminase by phosphate and borate were obtained at various concentrations of glutaminase and specific activity was plotted against time. The order of the activation with respect to enzyme concentration was determined using the differential equation:

$$\log \frac{dE'}{dt} = \log K + a \log E$$

where E = concentration of initial form of enzyme, E' = concentration of activated form of enzyme, K = constant and a = order of reaction. Thus a plot of log dE'/dt against log E has a slope equal to the order of the reaction. dE'/dt was obtained by measuring the initial rate of increase in specific activity and multiplying by the protein concentration. Since E' was measured in U/ml and E in mg/ml, K was not calculated. The equation assumes that only two forms of enzyme are present in the mixture.

Hill plots of the slow activation of soluble glutaminase

Time courses of the activation of soluble glutaminase by various concentrations of phosphate and borate were obtained and specific activity was plotted against time. The initial rate of activation (x) was measured (in arbitrary units) for each activator concentration. The initial rate of activation at saturating activator concentration (x_{max}) was estimated in the same way and the Hill coefficient and apparent K_A for the activation were derived from graphs of $\log [(x_{max} - x)/x]$ against log (activator concentration) [16].

RESULTS

The stability of glutaminase under assay conditions

The stability of pig brain soluble and membrane-bound glutaminases during assay was investigated as described in Fig. 1. The three continuous assay mixtures described in the Methods section were used. Three preparations of glutaminase were studied: (1) membrane bound glutaminase which had

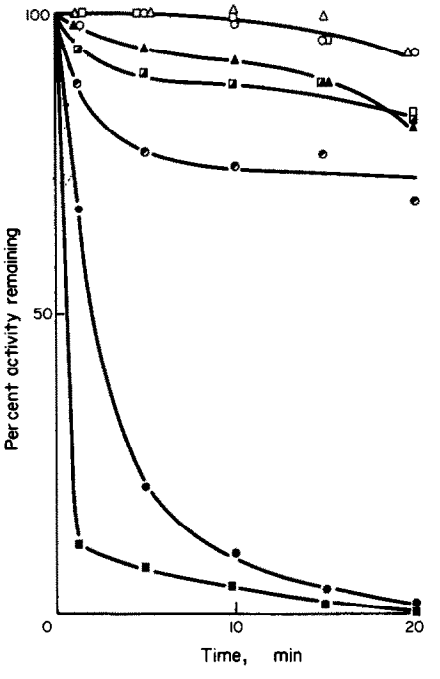

Fig. 1. Stability of glutaminase under assay conditions. Three preparations of glutaminase, the membrane-bound enzyme preparation (△), the Tris-HCl form of the soluble enzyme at a concentration of 0.25 mg/ml (□) and the phosphate borate form of the soluble enzyme at the same concentration (○), stored at 5°, were diluted 100-fold into the assay mixture used for the ammonium ion-sensitive glass electrode assay in the absence of glutamine (closed symbols), in the presence of 5 mM glutamine (open symbols), in the absence of glutamine but in the presence of 5 mM albizziin (divided symbols). These mixtures were incubated at 30° for the indicated times before 100 μl samples were withdrawn and assayed using the coupled assay for ammonium ions used with 20 mM sodium phosphate. Enzyme activities are expressed as percentages of the activity determined without pre-incubation. Details of the enzyme preparations and assays are given in the text.

been freed of soluble glutaminase and stored in 100 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.05 per cent 2-mercaptoethanol (2) pure soluble glutaminase stored in 100 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM DTT (the Tris-HCl form of the enzyme) (3) pure soluble glutaminase stored in 50 mM phosphate-15 mM borate-Tris buffer pH 7.4, 1 mM EDTA, 1 mM DTT (the phosphate-borate form of the enzyme). The results obtained with the ammonium ion-sensitive electrode assay mixture are shown in Fig. 1. The results were qualitatively similar for the other two assay mixtures. Soluble glutaminase stored in either buffer was less stable than membrane-bound glutaminase when diluted into assay mixtures in the absence of glutamine and time courses of inactivation are shown in Fig. 1. However, when 5 mM glutamine was added to the assay mixture before the glutaminase, all three preparations of glutaminase were stable for the first 5–10 min after dilution into it.

Albizziin is a reversible competitive inhibitor of soluble glutaminase. The K_i value was found to be 1.8 mM for soluble glutaminase preincubated in Tris-HCl buffer and 5.5 mM for soluble glutaminase preincubated in phosphate-borate-Tris-buffer (data not shown). It did not inhibit membrane-bound glutaminase. Albizziin was able to protect soluble glutaminase from inactivation caused by dilution into the assay mixture but not as effectively as glutamine (Fig. 1).

The specific activity and stability of soluble glutaminase stored in various buffers

The stability of soluble glutaminase was dependent on the composition of the buffer in which it was stored as illustrated in Fig. 2. After an initial lag, enzyme activity was lost in an apparently first-order manner. The enzyme was more stable when concentrated and when stored at 0–5° than at 30° [2].

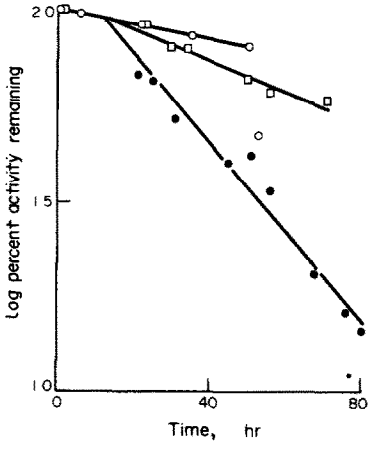

Fig. 2. Stability of soluble glutaminase during storage. Soluble glutaminase was stored at 5° and at a concentration of 0.83 mg/ml in 20 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 1 mM DTT (●), the same Tris buffer mixture plus 15 mM borate-NaOH buffer, pH 7.4, (□) or the same Tris buffer mixture containing 50 mM phosphate-15 mM borate-NaOH buffer, pH 7.4 (○). The enzyme was incubated in the buffer mixture for 12 hr before any measurements of activity were made. Activities were determined by the coupled assay for the production of ammonium ion used with 20 mM sodium phosphate. Other details were as described in the text.

The effect of dilution on the specific activity of soluble glutaminase

When soluble glutaminase was diluted without changing the composition of the buffer, the enzyme preparations in Tris-HCl and in sodium phosphate buffer lost activity rapidly whereas it was fairly stable in sodium borate and in phosphate-borate-NaOH buffer (Fig. 3). Comparing Figs 1 and 3 it can be seen that the phosphate-borate form of soluble glutaminase is much less stable to dilution when phosphate and borate are absent from the diluent.

On dilution the enzyme lost activity in an apparently first-order manner to reach a new lower specific activity. The loss of activity was greater at higher dilutions. As the Tris-HCl form of the enzyme aged, on storage at 5°, it became more unstable to dilution. The phosphate-borate form of the enzyme was much more stable, as illustrated in Fig. 2, and its properties changed much more slowly with ageing than did those of the Tris-HCl form of the enzyme. Except where indicated, soluble glutaminase was not stored in Tris-HCl buffer for more than 36 hr to reduce this effect of ageing on its properties. The effects of dilution were very similar at 0° and 30° (Fig. 7). The inactivation caused by dilution could not be prevented by keeping the total protein concentration constant with bovine serum albumin.

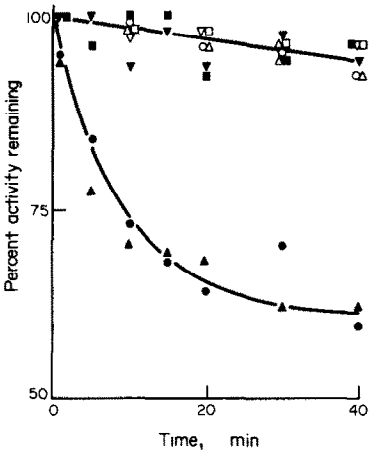

Fig. 3. The effects of dilution on the specific activity of soluble glutaminase. The effects of buffer composition. Soluble glutaminase was preincubated for 30 min at 30° at a concentration of 0.29 mg/ml in 20 mM Tris-HCl buffer, pH 7.4 containing 1 mM EDTA with no further additions (○), with the addition of 50 mM phosphate-NaOH buffer, pH 7.4, (△), with the addition of 15 mM borate-NaOH buffer, pH 7.4, (□) or with the addition of 50 mM phosphate-15 mM borate-NaOH buffer, pH 7.4 (▽). After this time the enzyme samples were either diluted 10-fold (closed symbols) or left undiluted (open symbols) and samples were withdrawn at the indicated times and assayed using the coupled assay for the production of ammonium ions used with 20 mM sodium phosphate. Activities are expressed as percentages of the values determined at the end of the first incubation.

Slow activation of soluble glutaminase by phosphate and borate ions

The effect of diluting soluble glutaminase in the Tris-HCl form, to different extents into 15 mM sodium borate is shown in Fig. 4. Soluble glutaminase was slowly activated by borate ions and the activation appeared to be greater at higher enzyme concentrations. This activation was stable to dilution into the assay mixture where the borate ions and enzyme were diluted to concentrations (1 mM and <1 μg/ml respectively) which were not high enough to permit time-dependent activation (see Fig. 4). The order of the reaction with respect to enzyme concentration was estimated, as described in the Methods section, from Fig. 5 and found to be 3.5.

The effect of borate concentration on the activation of a fixed concentration of soluble glutaminase in the Tris-HCl form by borate was studied and a Hill plot was constructed as described in the Methods section. This plot is shown in Fig. 6; the apparent K_a for borate was found to be 9.6 mM and the Hill constant was 1.8. As the enzyme aged it was activated to a lesser extent by borate.

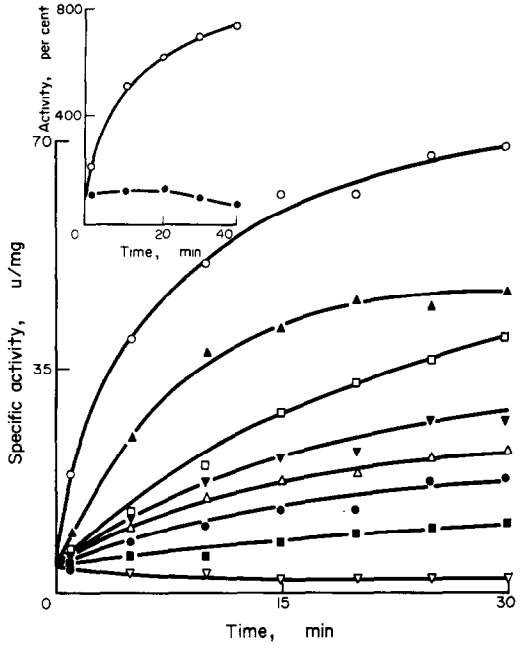

Fig. 4. The effects of enzyme concentration on the time-course of activation of soluble glutaminase in the Tris-HCl form by 15 mM borate. Soluble glutaminase at a concentration of 0.83 mg/ml in 20 mM Tris-HCl buffer, pH 7.4 containing 1 mM EDTA and 1 mM DTT was diluted into the same buffer mixture containing 15 mM borate-NaOH buffer, pH 7.4 and maintained at 30°. Samples were withdrawn at the indicated times and assayed using the coupled assay for the production of ammonium ions with 20 mM sodium phosphate. The final glutaminase concentrations were 0.415 (○), 0.332 (▲), 0.249 (□), 0.208 (▼), 0.166 (△, ●), 0.083 (■) and 0.008 (▽) mg/ml. *Inset*: Comparison of the time-courses of activation of enzyme samples in the Tris-HCl buffer mixture after dilution from an initial concentration of 0.558 mg/ml into the same buffer mixture containing 100 mM borate-NaOH buffer, pH 7.4, to give a concentration of 0.224 mg/ml. (○), freshly-prepared enzyme solution treated in this way (specific activity after dilution of 3.5 U/mg taken as 100 per cent); (●) the same enzyme preparation stored at 5° for 3 days before dilution (specific activity after dilution of 1.8 U/mg taken as 100 per cent).

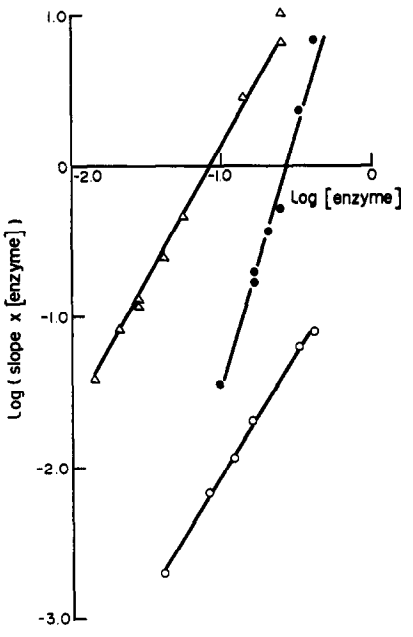

Fig. 5. Time-dependent activation of soluble glutaminase by phosphate and borate: order of reaction with respect to enzyme. The plots were constructed as described in the text, the slopes are expressed in arbitrary units and the glutaminase concentrations in mg/ml. Activation of soluble glutaminase in the Tris-HCl form by 15 mM borate (data obtained from Fig. 4) (●); activation of soluble glutaminase stored at 0.286 mg/ml in 20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT pH 7.4, by dilution into 50 mM phosphate (time-courses not shown) (△); activation of soluble glutaminase, stored at 0.83 mg/ml in 20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 15 mM borate-NaOH, pH 7.4, by dilution into 100 mM phosphate (time-courses not shown) (○).

The slow activation of soluble glutaminase in the Tris-HCl form by sodium phosphate was examined in the same manner as that described for the borate activation. From time courses of activation at various enzyme concentrations and at various phosphate concentrations, the data shown in Fig. 5 allowed the order of the activation reaction with respect to enzyme concentration to be calculated to be 1.9 and the data shown in Fig. 5 gave values for the apparent K_a for phosphate of 32 mM and for the Hill constant of 2.2. The maximum degree of activation given by phosphate and borate was found to be about 10-fold in each case.

As shown previously by Svenneby [11], temperature has a marked effect on the activation of soluble glutaminase by borate and by phosphate. When soluble glutaminase in the Tris-HCl form was incubated with phosphate or borate as described previously, but at 0° rather than at 30°, no activation was observed although the enzyme was more stable to dilution than it would have been in the absence of phosphate or borate. When the incubations were

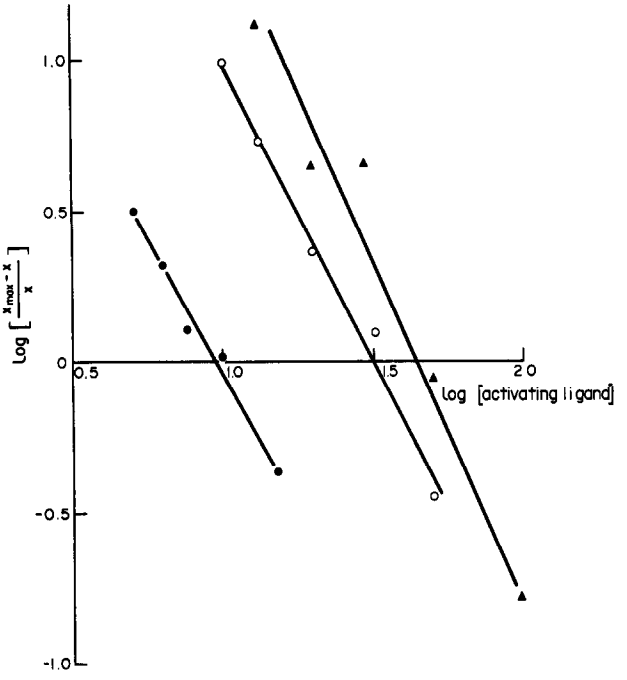

Fig. 6. Hill plots of the slow activation of soluble glutaminase by phosphate and borate. The plots were constructed as described in the text; the slopes (x) are expressed in arbitrary units and the ligand concentrations in mM. Activation of soluble glutaminase stored at 0.83 mg/ml in 20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.4 by dilution at 30° into the same mixture containing borate buffer to give a final concentration of 0.332 mg/ml (●), activation of soluble glutaminase, stored at 0.449 mg/ml in 20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT pH 7.4, by dilution at 30° into the same mixture containing phosphate buffer to give a final concentration of 0.359 mg/ml (○), activation of soluble glutaminase, stored at 0.83 mg/ml in 20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 15 mM borate-NaOH, pH 7.4, by dilution at 30° into the same mixture containing phosphate buffer, pH 7.4, to give a final concentration of 0.332 mg/ml (▲). The time-courses were determined in a similar manner to that described in Fig. 4.

then warmed to 30° activation proceeded normally. The progress of the activation at 30° could be halted at any time, but not reversed, by lowering the temperature to 0°.

The slow activation of soluble glutaminase in the borate form by sodium phosphate was examined in the same manner as that described for the activation of the Tris-HCl form of the enzyme. The time courses of activation at various concentrations of glutaminase are not illustrated but the replot is shown in Fig. 5. The order of the reaction with respect to enzyme concentration was found to be 1.7. The time courses of activation at different concentrations of phosphate are not shown but the replot is shown in Fig. 6. The apparent K_a for phosphate was calculated to be 43 mM and the Hill constant was 2.4. These values were very similar to those obtained for the activation of the Tris-HCl form of soluble glutaminase by phosphate, but the maximum activation of the borate form of the enzyme by phosphate was less than two-fold.

The slow activation of the enzyme by phosphate and borate was reversible (Fig. 1; see also [17]) and it was not caused by the increase in ionic strength or by the Na^+ ions since no activation was observed when soluble glutaminase in the Tris-HCl form was incubated for 30 min at 30° with 50 mM NaCl or 50 mM NH_4Cl.

The effect of glutamate on the specific activity of soluble glutaminase

When soluble glutaminase in the Tris-HCl form was diluted into a solution of glutamate there was a very rapid fall in the specific activity which then levelled off at a new and lower value (Fig. 7). The extent of the fall in activity was greater at high enzyme dilutions, high glutamate concentrations and high temperatures. The rate of fall of the activity was too rapid to analyse in detail.

Membrane-bound glutaminase

The specific activity of membrane-bound glutaminase was not affected in a time-dependent manner by 50 mM phosphate, 20 mM borate, 50 mM NaCl, 50 mM NH_4Cl or 100 mM glutamate during a 30 min incubation at 30°. The enzyme was not inactivated by dilution (Fig. 1). It was slightly more stable in phosphate-borate-Tris buffer than in Tris-HCl buffer.

DISCUSSION

The loss of activity that occurs when soluble glutaminase is diluted can be prevented by the presence of glutamine and some protection was also afforded by the competitive inhibitor albizziin. In the case of the phosphate-borate form of the enzyme

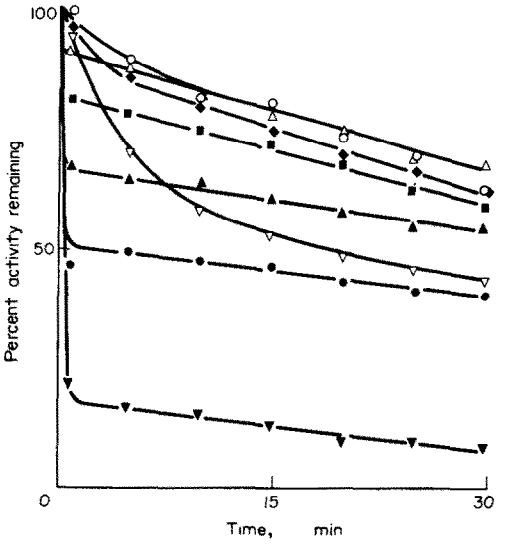

Fig. 7. The effects of glutamate on the activity of soluble glutaminase after dilution. Soluble glutaminase at a concentration of 0.37 mg/ml in 20 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 1 mM DTT was diluted into the same buffer, with or without the addition of sodium glutamate at either 0 or 30°. The time-courses of activity change were determined as described in Fig. 1. Glutaminase incubated at 0.2 mg/ml at 0° with (■) and without (◆) 2 mM glutamate, glutaminase incubated at 0.006 mg/ml at 0° with (▼) and without (▽) 2 mM glutamate, glutaminase incubated at 0.2 mg/ml at 30° with 0 (○), 0.5 (△), 1.0 (▲) and 2.0 (●) mM glutamate.

dilution results in a reversal of the activation by these ions in addition to the dilution-dependent activity loss. Glutamine prevents both these effects (Fig. 1) allowing the enzyme to retain the activated state caused by phosphate plus borate even when these ions had been diluted to concentrations at which they were incapable of activating soluble glutaminase. The rate of inactivation of the enzyme in the presence of glutamate was very rapid but, again, the presence of glutamine protected the enzyme activity. In contrast Svenneby *et al.* [12] found that glutamine protected the Tris-HCl form of soluble glutaminase from pig kidney against inactivation by dilution but did not protect the phosphate-borate form.

The stability of glutaminase during storage depended upon the buffer used; the enzyme being more stable when stored in phosphate-borate or borate buffers than when it was stored in Tris-HCl buffer. As shown in Fig. 3. enzyme preparations stored and diluted in phosphate-borate or borate buffers were more stable than those stored and diluted in Tris-HCl or phosphate buffers, where the enzyme lost activity over a period of about 20 min after dilution, after which time slower inactivation occurred at a rate similar to that of the undiluted enzyme. The time-courses of inactivation due to dilution were similar at 0 and 30° but the extent of the initial rapid fall in activity was found to depend on the final enzyme concentration.

Decreases in the stability and sensitivity to activation of the preparation of soluble glutaminase stored in Tris-HCl buffer could complicate studies on the kinetics of the activation of this form of the enzyme by phosphate and borate. Svenneby [11] found great variability between different preparations of pig brain glutaminase in this respect and the pig kidney enzyme was found to be desensitized to activation by bromothymol blue by such storage [4] or mild heating [6]. In order to minimize these effects the enzyme was stored in phosphate borate buffer and transferred to Tris-HCl buffer, by gel-filtration or dialysis overnight, before use.

Kinetic studies of the time-courses of activation of the Tris-HCl form of the enzyme by borate showed the order of the reaction to be 3.5 with respect to enzyme concentration, consistent with the activation process depending on the aggregation of the enzyme [2, 17]. The dependence of the activation on the borate concentration gave a Hill constant of 1.8 indicating the existence of more than one binding site for this ligand. Since the binding of borate to the Tris-HCl form of the soluble enzyme is likely to precede the slow activation process this result would be consistent with this form of the enzyme being a dimer (G. A. Nimmo, Ph.D. Thesis, University of Cambridge, 1975) or a trimer [18]. The activation of the enzyme by phosphate gave an order of reaction with respect to enzyme concentration of 1.9 and a Hill constant for the activator of about 2 which would be consistent with the suggestion that this ligand causes aggregation of two molecules of the Tris-HCl form of the enzyme [11, 17]. The activation of the borate form of the enzyme by phosphate gave similar values for the order of reaction and Hill constant to those determined for the activation of the Tris-HCl form by this ligand although the maximum activation obtained was quite small indicating that the effects of phosphate and borate are not simply additive. The similar K_a values for phosphate activation of the two forms of the enzyme would suggest that borate and phosphate bind to different sites on the enzyme so that there is no competition between these ligands. This would also be in accord with the ability of phosphate to cause further aggregation of the borate form of the enzyme [4]. A Hill constant of 2 was also observed for the activation of glutaminase in rat kidney mitochondria by Kovacevic *et al.* [19]. These workers, observed no time-dependent effects and ascribed this to the high protein concentrations present. With whole mitochondria however the predominant activity might be that of the membrane-bound form (see e.g. [1]) which does not show such effects.

The interpretation of these results could be complicated if there were more than two forms of the enzyme present in solution since, as described earlier, the analysis assumed that only two forms (the Tris-HCl form and the borate or phosphate form, or the borate form and the phosphate-borate form) were present during the activation. The possible heterogeneity of the aggregates formed [2] (G. A. Nimmo, Ph.D. Thesis, University of Cambridge, 1975) and the occurrence of the reaction in discrete steps would result in the order of the reaction with respect to enzyme concentration giving an underestimate of the number of protomers contributing to the aggregated form. Instability of the enzyme could also complicate the time-courses of activation. However under the conditions used here phosphate and borate gave a considerable stabilizing effect even

at low enzyme concentrations where no significant activation was observed (see Figs 3 and 4).

These time-dependent effects necessitate great care in assay procedures to ensure that the true activity of the enzyme is to be measured. The dilution-dependent loss of activity can be prevented by the presence of glutamine. Thus it is necessary to start the assay by the addition of enzyme to the assay mixture containing glutamine rather than by the addition of glutamine. Time-dependent changes in the properties of the enzyme stored in Tris-HCl buffer could also lead to variability of the results obtained and storage in this buffer should be avoided. The slow activation of the enzyme by phosphate and borate only occurred significantly in rather concentrated solutions ($\geq$ 0.02 mg/ml) and the high specific activity of the enzyme would usually require the use of much lower enzyme concentrations for assay. Considerably greater concentrations have, however, been used in some kinetic studies using discontinuous assays (see e.g. [11, 18]) and these effects might well have affected the determined rates in such cases. In contrast to these effects the relative stability of the membrane-bound enzyme and the lack of observable time-dependence in its activation make assessment of its true activity a more straightforward matter.

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