Glutaminase from Pig Renal Cortex

II. ACTIVATION BY INORGANIC AND ORGANIC ANIONS

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SUMMARY

The Tris-HCl and phosphate-borate forms of glutaminase (EC 3.5.1.2, L-glutamine amidohydrolase) are both activated and protected from inactivation by phosphate and carboxylic acids. The Tris-HCl enzyme is less sensitive to this activation, but more sensitive to glutamate inhibition than the phosphate-borate enzyme. Plots of activity against concentration of activating anions are generally sigmoidal, and plots against phosphate become more sigmoidal by addition of glutamate. However, such plots for the phosphate-borate enzyme become hyperbolic in the presence of α-ketoglutarate.

In addition to spontaneous activation by phosphate and carboxylic acids, assumed to be of allosteric nature, a time-dependent activation has been shown at higher protein concentrations. The time-dependent activation occurs following incubation of the Tris-HCl enzyme with phosphate or phosphate plus borate. The time-dependent activation in the presence of phosphate plus borate changes the properties of the Tris-HCl enzyme so that they become similar to those of the phosphate-borate enzyme. It is suggested that time-dependent activation reflects a transition of the Tris-HCl enzyme to higher molecular forms.

EXPERIMENTAL PROCEDURE

L-Glutamine and L-glutamic dehydrogenase (EC 1.4.1.2) (type II) were products of Sigma. The other reagents were of highest purity, analytical grade.

In the preceding paper (1), a procedure was described which leads to a highly purified preparation of pig kidney glutaminase (EC 3.5.1.2, L-glutamine amidohydrolase). The enzyme was found to exist in different molecular forms and to have allosteric properties.

Two molecular forms, a Tris-solubilized and a phosphate-borate-precipitated form, have been extensively investigated. Phosphate and carboxylic acids are likely to be allosteric activators with different affinities for the two enzyme forms. Furthermore, the Tris-HCl enzyme is activated by phosphate and phosphate plus borate in another way, dependent on time and protein concentration, whereby the properties of the enzyme are changed.

RESULTS

Effect of Phosphate and Carboxylic Acids When Added to Assay System—We have previously reported that the activity of both kidney (3) and brain (4) glutaminase was greatly influenced by carboxylic acids. This effect has later been observed by others, with kidney mitochondria (5) or brain extract (6) as the source of enzyme. As shown in Table I, carboxylic acids activated both T-form and the P-B-form, although the latter was more sensitive to activation. Thus, activation of the T-form by carboxylic acids was only 5 to 10% of the activation due to phosphate at the same concentration, whereas phosphate and carboxylic acids activated the P-B-form to the same extent.

When high phosphate concentrations were used, phosphate activation was not additive to that of carboxylic acids. The activation pattern may be used to distinguish the forms of glutaminase described here from the glutaminase isoenzyme of Katanuma, Tomino, and Nishino (7), which is stimulated by maleate only and not by phosphate. In contrast to the P-B-form, the T-form has very low or no residual activity when assayed in the absence of activators.

It has previously been shown that the activating dye bromothymol blue protects glutaminase from inactivation by heavy metals, p-chloromercuribenzoate, and chloride (4). As shown in Table II, phosphate and α-ketoglutarate also protect glutaminase from inactivation. Highly diluted glutaminase was previously incubated at 23° for 5 min before the assay, with and without glutamine, α-ketoglutarate, and phosphate. α-Ketoglutarate and phosphate protect both enzyme forms whereas the substrate, glutamine, when added without phosphate and α-ketoglutarate, protects the T-form particularly. When the enzyme is incubated with glutamine, phosphate, and α-ketoglutarate prior to assay, activity is only slightly reduced compared to that of nonincubated enzyme.

In one experimental series, glutaminase (P-B-form) was previously incubated in borate buffer containing EDTA. Borate inhibited enzyme activity slightly, but inactivation did not
Activation of glutaminase by phosphate and carboxylic acids

Glutaminase was assayed by chromatographic determination of glutamate formation following incubation for 5 min (23°, pH 8.0) (1). The incubation mixture, 1 ml, contained 40 mM L-glutamine, 0.5 mM EDTA, and, where indicated, 1.5 μg of Tris-HCl enzyme, 0.5 μg of phosphate-borate enzyme, and 50 mM phosphate or organic anions (or both) of the sodium salts.

<table>
<thead>
<tr>
<th>Additions</th>
<th>T-form</th>
<th>P-B-form</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pi⁺</td>
<td>Pi⁻</td>
</tr>
<tr>
<td>None</td>
<td>129</td>
<td>0</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>121</td>
<td>4</td>
</tr>
<tr>
<td>Succinate</td>
<td>129</td>
<td>7</td>
</tr>
<tr>
<td>Malate</td>
<td>111</td>
<td>4</td>
</tr>
<tr>
<td>Malonate</td>
<td>138</td>
<td>16</td>
</tr>
<tr>
<td>Maleate</td>
<td>136</td>
<td>19</td>
</tr>
<tr>
<td>Citrate</td>
<td>116</td>
<td>8</td>
</tr>
</tbody>
</table>

* Nanomoles of glutamate formed per min.

Protection from inactivation of glutaminase

Highly diluted glutaminase was incubated for 5 min at 23° and pH 8.0 and then assayed. Where indicated, the preliminary incubation medium contained 1.5 μg per ml of Tris-HCl enzyme, 0.5 μg per ml of phosphate-borate enzyme, 8 mM L-glutamine, 20 mM α-ketoglutarate, and 50 mM sodium phosphate. In one experimental series the preliminary incubation medium also contained 10 mM sodium borate. The assay was performed at 23° and pH 8.0 by the spectrophotometric technique (1). The assay medium, 1 ml, contained 8 mM L-glutamine, 20 mM α-ketoglutarate, 50 mM sodium phosphate, 0.5 mM EDTA, 0.1 mM NADH, and 1 i.u. of L-glutamic dehydrogenase.

<table>
<thead>
<tr>
<th>Prior incubation with</th>
<th>T-form</th>
<th>P-B-form</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alone</td>
<td>With borate</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>40</td>
<td>74</td>
</tr>
<tr>
<td>Glutamine</td>
<td>54</td>
<td>10</td>
</tr>
<tr>
<td>Pi⁻</td>
<td>72</td>
<td>47</td>
</tr>
<tr>
<td>Glutamine + α-ketoglutarate</td>
<td>64</td>
<td>102</td>
</tr>
<tr>
<td>Pi⁻ + α-ketoglutarate</td>
<td>82</td>
<td>79</td>
</tr>
<tr>
<td>Glutamine + Pi⁻</td>
<td>69</td>
<td>118</td>
</tr>
<tr>
<td>Glutamine + Pi⁻ + α-ketoglutarate</td>
<td>115</td>
<td>126</td>
</tr>
</tbody>
</table>

* Nanomoles of NH₃ formed per min.

Fig. 1. The effect of α-ketoglutarate on the activity of the Tris-HCl and phosphate-borate enzymes. Glutaminase was assayed by paper chromatographic determination of glutamate (1). Assay conditions were as in Table I, except that the incubation time was 30 min. Other additions are indicated: Δ, 0.1 mM phosphate; ○, 20 mM α-ketoglutarate; ○, phosphate plus α-ketoglutarate; △, control without phosphate or α-ketoglutarate. A, phosphate-borate enzyme. B, Tris-HCl enzyme. Enzyme activity is expressed as micromoles of glutamate formed per min.

Fig. 2. The phosphate activation of the Tris-HCl enzyme compared with that of the phosphate-borate enzyme. Glutaminase was assayed by determination of ammonia formation (2). Experimental conditions were as in Table I, except that varying amounts of phosphate were added. Where indicated, the assay mixture also contained 20 mM α-ketoglutarate (α-Ket) and 4 mM glutamate (Glu). Enzyme activity is expressed as micromoles of ammonia formed per min.

As described above, activation of both enzyme forms by high concentrations of phosphate was not observed in the case of the T-form.
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Phosphate concentrations (50 mM) is not additive to that of carboxylic acids. However, when low phosphate concentrations (7.5 mM) are used, the activation of the T-form by organic anions is additive (Fig. 3). Plots of activity against concentration of activator were sigmoidal for both enzyme forms (Figs. 2 and 3), and plots against phosphate became more sigmoidal by addition of the inhibitor glutamate (only shown for the T-form in Fig. 2). This supports the view that both enzyme forms are allosteric proteins. However, such plots for the P-B-form became hyperbolic in the presence of α-ketoglutarate, in contrast to plots for the T-form (Fig. 2). With no α-ketoglutarate present, the apparent $K_m$ for phosphate decreased with the phosphate concentration and reached a minimal value of 20 mM for the T-form and 3 mM for the P-B-form.

The different effects of glutamate on the activity of the T-form and P-B-form of glutaminase are shown in Fig. 4. With high concentrations of phosphate (50 mM) in the assay, the T-form was considerably more sensitive to glutamate inhibition than the P-B-form. However, in the presence of low concentrations of phosphate (less than 10 mM), the sensitivity of the
P-B-form to glutamate inhibition increased somewhat. Reciprocal plots of activity against glutamine concentration in the presence of 50 mM phosphate and varying amounts of glutamate were linear and did not intersect at one point on the ordinate.

Time-dependent Activation of T-form by Phosphate and Phosphate plus Borate—In the experiments shown in Fig. 5, the T-form was previously incubated with phosphate buffer containing borate, and samples to be assayed were withdrawn at the times indicated. It should be noted that the protein concentration of the preliminary incubation mixture was high, i.e. 70 μg per ml, whereas the protein concentration of the assay mixture was kept at the usual level of 0.3 μg per ml. The phosphate concentration and ionic composition of the assay mixture were also kept constant. The specific activity increased about 3-fold by preliminary incubation with phosphate plus borate under optimal conditions. This change is similar to that observed when the T-form is converted to the P-B-form (1). Moreover, the T-form is practically inactive when phosphate or other activator is not added to the assay, whereas the residual activity is increased considerably by preliminary incubation with phosphate and borate. This increase is also similar to that observed when the T-form is converted to the P-B-form. It may be seen from Fig. 5 that the change in specific activity followed pseudo-first order kinetics. The rate constants were $k (23^\circ) = 49$ sec$^{-1}$, and $k (0^\circ) = 4$ sec$^{-1}$, and the half-times for the reaction were $4 (23^\circ) = 51$ sec and $4 (0^\circ) = 10.6$ min. The initial rate was found to be of the same order of magnitude when the T-form was previously incubated with phosphate alone at $23^\circ$, but the curve leveled off earlier. No appreciable change in specific activity was found upon incubation with phosphate at $0^\circ$ prior to assay. In contrast to phosphate, incubation with malonate did not change the specific activity at $23^\circ$ in spite of the fact that malonate activated the enzyme when added to the assay system (1). The sedimentation coefficient measured with

**Fig. 7.** The effect of phosphate and borate on time-dependent increase in specific activity. Experimental conditions and assay were as described in Fig. 5 except that the Tris-HCl enzyme was incubated for 10 min at $23^\circ$: $\Delta$, with varying concentrations of phosphate; and $\bigcirc$, with varying concentrations of borate and 70 mM phosphate. Enzyme activity is expressed as micromoles of ammonia formed per min.

**Fig. 8.** Glutamate inhibition of the time-dependent increase in specific activity. Experimental conditions and assay were as in Fig. 5 except that the Tris-HCl enzyme was incubated for 10 min at $23^\circ$ with phosphate buffer of varying concentration with $\Delta$ or without $\bigcirc$, 2.5 mM glutamate.

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stant, shape of pH curve, activation by phosphate and carboxylic acids, inhibition by glutamate, number of sulfhydryl groups (9), and appearance in the electron microscope (9).

It has been shown that phosphate activates the T-form in two ways. In dilute enzyme solutions, a spontaneous activation occurs which is dependent on phosphate concentration. In more concentrated solutions, a time-dependent increase in specific activity is observed.

The first type of activation is shown by plots of velocity against concentration of phosphate or organic anions added to the assay. Such plots are sigmoidal for both enzyme forms which, in this respect, behave as allosteric enzymes. The activation of both P-B-form and the T-form by carboxylic acids is not additive to that of phosphate when the latter is present in high concentrations. Thus, phosphate and carboxylic acids are likely to compete for the same allosteric site on both enzyme forms, although these compounds have a much higher affinity for the site on the P-B-form than that on the T-form. In contrast to the T-form, α-ketoglutarate may bind to an allosteric site on the P-B-form, resulting in the loss of the allosteric property.

The second, time-dependent type of activation by phosphate and phosphate plus borate is also a function of the concentration of protein, and we assume that time-dependent activation may be caused by transition of the T-form to higher molecular forms. The P-B-form does not show time-dependent activation, while activation of the T-form by phosphate is further enhanced by borate. In the presence of phosphate and borate, the specific activity increases to the same extent as by the standard method for conversion of the T-form to P-B-form. In either case, residual activity, as measured without phosphate in the assay mixture, also increases considerably. Moreover, the T-form has globular structure, as shown by electron microscopy, whereas the P-B form appears as large helices (9). Recent studies have shown that conversion to helical structure occurs during the first minutes following addition of phosphate and borate to the T-form, provided that the conditions are similar to those for time-dependent activation.

The activation by α-ketoglutarate and ammonia (1) and inhibition by glutamate are of particular interest because these compounds participate in the glutamic dehydrogenase reaction. When glutamic dehydrogenase is directed to form α-ketoglutarate and ammonia, glutaminase may be stimulated and vice versa. Both enzymes are located in the mitochondria, which allows regulation of glutaminase by the glutamic dehydrogenase reaction as a possibility.

REFERENCES