Involvement of essential cysteine and histidine residues in the activity of isolated glutaminase from tumour cells

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Abstract

The pH dependence of the phosphate-activated glutaminase isolated from Ehrlich tumour cells suggests a functional role for two prototropic groups with apparent pKₐ of 9.3 and 7.7 at the active site of the protein; these pKₐ values are compatible with cysteine and histidine residues, respectively. This possibility was investigated by chemical modification studies of the purified enzyme. N-Ethylmaleimide fully inactivated the purified glutaminase; the reaction order was very close to 1.0, suggesting that N-ethylmaleimide modifies glutaminase at a single essential site. Spectrophotometric studies of the isolated protein treated with diethyl pyrocarbonate indicate that two histidine residues are modified. Since glutaminase is loosely associated to the inner mitochondrial membrane, modification experiments were also carried out using mitochondrial membrane fractions. N-Ethylmaleimide and diethyl pyrocarbonate gave similar results in mitochondria membrane-bound enzyme to those obtained with purified enzyme. Glutamate, which behaves as a competitive inhibitor of the enzyme, partially protected the inactivation caused by N-ethylmaleimide in membrane-bound experiments. The results suggest the existence of a critical histidine residue(s) in the tumour glutaminase, and strongly support the notion that a cysteine residue, which is located at (or near) the active site, is involved in the catalytic mechanism as well. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Glutaminase; Enzyme inactivation; Cysteine; Histidine residue

1. Introduction

In mammals, the mitochondrial phosphate-activated glutaminase, (PAG, L-glutamine amidohydrolase, EC 3.5.1.2) appears as two different isoenzymes, liver (PAG-L) and kidney (PAG-K) types. The first isoform is present in the adult liver; ammonia and inorganic phosphate are the only modulators of activity of physiological significance [1]; the cDNA of this enzyme has been recently cloned [2]. The PAG-K isoenzymes, mainly found in kidney, brain, foetal liver and small intestine, are immunologically related and exhibit similar molecular and kinetic properties [3–5]; the cDNA of glutaminase from rat kidney has been cloned [6]. In addition to their activation by various polyvalent anions, PAG-K isoenzymes are also competitively inhibited by glutamate [7].

In contrast with the liver and kidney enzymes, very little is known about tumour glutaminases, even though their relevance on the growth rate of tumour and proliferating tissues have been largely recognised...
The enzyme from Ehrlich ascites cells has been isolated from tumour mitochondria and showed a kinetic behaviour, with respect to glutamine and phosphate, characteristic of the PAG-K type [10,11]. A long-term regulation of this enzyme has been described, in such a way that maximum activities and mRNA levels are achieved during the exponential growth phase of tumour cells [12]. Aledo et al. [13] have very recently confirmed the location and membrane topography of the tumour enzyme; the enzyme is loosely associated to the inner mitochondrial membrane.

It has been suggested that in vivo PAG regulation might be achieved varying the phosphate and glutamate concentrations [4,14]; however, the details of molecular mechanism of the regulation are not completely understood. Chiu and Boeker [15] pointed out that glutamine and glutamate bind preferentially to the same site, but at two different conformations elicited by the activator phosphate. In a posterior kinetic study, Shapiro et al. [16] concluded that binding at the same glutamine site causes glutamate inhibition of the membrane-associated renal PAG.

Several sulfhydryl-specific reagents inactivate the liver and kidney PAG proteins. This fact has been used to prove the association of glutaminase with the inner membrane of intact mitochondria [13,17–19]. However, these thiol groups have not yet been well characterised in terms of either its location or functional role. Moreover, to our knowledge, there is no report about essential histidine residues in PAG enzymes.

The aim of this work was to obtain insight into amino acid residues implicated in active site and catalytic mechanism of the tumour PAG enzyme by evaluation of the data obtained by chemical modification of the isolated enzyme and membrane-bound enzyme, and ligand protection studies. The results presented here strongly suggest the implication of cysteine and histidine residues in the active site.

2. Materials and methods

2.1. Materials

L-[3,4-3H(N)]Glutamine (specific activity 1.79 TBq mmol\(^{-1}\)) and Aquasol-2 scintillation cocktail were supplied by NEN (Dreieich, Germany). Amino acids, NEM, DEPC, N-phenylmaleimide were purchased from Sigma (St. Louis, MO, USA). BSA (fraction V), GDH, ADP and NAD\(^+\) were products of Boehr-inger Mannheim (Mannheim, Germany). N-(1-Pyr-ene)maleimide was from Fluorescent Probes (Eugene, OR, USA). The Dowex AG 1-X8 anionic exchange resin was a product of Bio-Rad (Brussels, Belgium). All the other reagents were of the highest purity commercially available. Ultra pure water generated by a Milli-RO 60 coupled to Milli-Q water purification system (Millipore, USA) was used to prepare all the aqueous solutions.

2.2. Ehrlich ascites cells

A highly malignant hyperdiploid Lettré strain was maintained in 2-month-old female albino Swiss mice, OF1 (SPF Ico). The cells were harvested from the peritoneal cavity about 10 days after the mice were inoculated with 5 \( \times 10^6 \) cells, as described elsewhere [10]. The animals received standard Panlab food with tap water ad libitum and were kept at 20 \( \pm \) 2\(^\circ\)C, with light from 08.00 to 20.00 h. The life span of the animals after inoculation was 16 \( \pm \) 1 days.

2.3. Isolation of mitochondria

Ascites-tumour-cell mitochondria were isolated as previously described [20]. Mitochondria were suspended at 20 mg ml\(^{-1}\) in 20 mM Tris-HCl, 210 mM mannitol, 70 mM sucrose and 1 mM EGTA, pH 8.0 (buffer A) and stored at \(-80\)\(^\circ\)C until use.

2.4. Isolation of phosphate-activated glutaminase from mitochondria of Ehrlich ascites tumour cells

The enzyme was purified to apparent homogeneity according to the procedure described by Segura et al. [11]. The purified enzyme had a specific activity of about 32 U mg protein\(^{-1}\). The purified enzyme was suspended in 20 mM Tris-HCl, 1 mM EDTA and 100 mM sucrose pH 8 (buffer B) and stored at \(-20\)\(^\circ\)C.

2.5. Glutaminase assay

PAG activity was measured as described by Aledo...
et al. [20], with the glutamate dehydrogenase reaction as an auxiliary system. To study the effect of pH on isolated glutaminase activity different buffered media at 50 mM final concentration were used: MES-KOH (pH 6.0 and 6.5), HEPES-KOH (pH 7.0 and 7.5), Tris-HCl (pH 8.0, 8.5 and 9.0) and \( \text{CO}_2/\text{HCO}_3^- \) (pH 9.5, 10.0, and 10.5). Protein concentrations were determined by a method recommended for membrane protein [21].

2.6. Chemical modification by N-ethylmaleimide

The rate of PAG modification by NEM was determined by monitoring the inactivation of the glutaminase activity using both isolated enzyme or mitochondrial membrane-bound enzyme. For purified protein inactivation the enzyme was incubated at 25°C with 0.1, 0.25, 0.5 and 0.75 mM NEM for different times in a volume of 240 \( \mu \)l in 1.0 mM buffer B pH 8.0. After a desired time, the reaction of the enzyme with NEM was stopped by quenching the mercurial with 20-fold concentration of \( \beta \)-mercaptoethanol to a final volume of 250 \( \mu \)l. Afterward, the glutaminase activity was assayed. In order to inactivate the mitochondria membrane-bound enzyme, freeze–thawed mitochondria at 4.0 mg ml\(^{-1}\) protein concentration in buffer A were incubated at 25°C with NEM (0.5-4 mM). Aliquots were withdrawn after fixed times, quenched with 0.2 M \( \beta \)-mercaptoethanol and assayed for PAG activity. To prevent mitochondrial respiration, 1.2 \( \mu \)g ml\(^{-1}\) antimycin A and 20 \( \mu \)g ml\(^{-1}\) oligomycin were included in the incubation media. Control mitochondria without being exposed to NEM were subjected to the whole process. No significant differences in PAG activity were found between control mitochondria and untreated freeze–thawed mitochondria.

The pseudo-first-order rate constants for inactivation were obtained by a linear least-squares fit of the plot of ln (% remaining activity) versus time. For protection experiments, mitochondria (4 mg ml\(^{-1}\)) in buffer A were preincubated for 5 min at 25°C with the indicated concentration of effectors. Then, NEM at 1.0 mM final concentration was added during an additional 5 min incubation period. The reaction was stopped with \( \beta \)-mercaptoethanol and the mitochondria were washed twice with 4 vols. of buffer A, to assure that no effector was present in the uterion activity assay. Incubating control samples with saturating concentrations of phosphate, or glutamine or glutamate checked this fact. After washing as above, the activity was determined by three different assays: (a) glutamine omitted; (b) phosphate omitted; and (c) standard assay as described above. PAG activity was only detected when glutamine and phosphate were simultaneously present in the standard assay; thus, the possibility that the effectors influence the PAG activity assay must be ruled out.

2.7. Chemical modification with diethyl pyrocarbonate

The ethanol diethyl pyrocarbonate (DEPC) solutions were always extemporaneously prepared, just before use; the final concentration of ethanol in the reaction mixture never exceeded 0.75% (v/v). For inactivation of the isolated enzyme, 9.2 mU of the enzyme were preincubated in buffer HEPES-KOH pH 7.2, for 10 min at 25°C at different DEPC concentrations in a final volume of 75 \( \mu \)l.

For glutaminase inactivation using mitochondria, the mitochondria suspension was incubated at different times in 210 mM mannitol, 70 mM sucrose, 5.0 mM HEPES-KOH and 1.0 mM EGTA pH 7.2, supplemented with 1.2 \( \mu \)g ml\(^{-1}\) antimycin A and 20 \( \mu \)g ml\(^{-1}\) oligomycin. The extent of inactivation was determined by measuring the residual PAG activity on aliquots removed from the reaction mixture and washed twice with 4 vols. of buffer A. Control incubations without DEPC were run in parallel. The activity of the experimental incubation solution (A) was divided by the activity of the control incubation (A\(_c\)) to correct for any small activity loss not due to the inactivating reagent.

2.8. Glutamate inhibition experiments

For the kinetic experiments of glutamate inhibition in mitochondria the radioactive assay of Shapiro et al. [16] was followed with some modifications. A reaction mixture of 60 \( \mu \)l containing 0.1 \( \mu \)Ci of \( \text{L-[3,4}^3\text{H(N)} \) glutamine, 0.2 mM EDTA, 20 mM Tris-acetate pH 8.0, 30–50 \( \mu \)g of mitochondrial protein and the concentrations of glutamine, phosphate and glutamate indicated in the figure legends, were incubated at 37°C for 10 min. Samples were frozen in liquid nitrogen. They were thawed by adding 1 ml
of 30 mM glutamine, 3 mM glutamate in water adjusted to pH 7.0 with NaOH, and applied to Dowex AG 1-X8 columns (1.2 × 1.5 cm) pre-equilibrated with water. After washing with 4 × 1 ml of 30 mM glutamine in water, the [3H]glutamate was then eluted with 2 × 2 ml of 2 N acetic acid. To these samples, 15 ml of Aquasol-2 scintillation cocktail were added and radioactivity determined in a LKB Rack-Beta scintillation counter. Blanks with mitochondria omitted were always run in parallel. Each point represents the mean of duplicate determinations.

3. Results

3.1. Dependence of pH of isolated phosphate-activated glutaminase activity

Fig. 1 shows the pH dependence of the apparent $V_m$ of purified glutaminase at different pH values. The shape of the curve is consistent with a diprotic enzyme model in which the successive $pK_a$ values of the amino acid residues of the enzyme are closer than 3.5 pH units. Since the $pK_a$ values of the amino acid residues implicated in the catalysis are separated by less than 2 pH units, the procedure described by Segel [22] must be used to determine the $pK_{a1}$ and $pK_{a2}$ values. Taking into account that the observed $V_m$ at pH 8.5 is a fraction of the total enzyme $[E_0]$, present as $[EH^-]$, the catalytic form of the enzyme, the value of $pK_{a1}$ could be calculated according to:

$$1[H^+]_{1/2} + 2[H^+]_{1/2} = K_{a1} + 4[H^+]_{opt}$$

Where $1[H^+]_{1/2}$ and $2[H^+]_{1/2}$ are the experimental values of proton concentrations at which the apparent $V_m$ values are the half value of $V_m$ at the optimum ($V_{m_{opt}}$); $[H^+]_{opt}$ is the proton concentration at the optimum, and $K_{a1}$ is the dissociation constant of the amino acid residue. Since the concentration of proton in the optimum is $[H^+]_{opt} = \sqrt{K_{a1}K_{a2}}$, the calculated $pK_{a1}$ and $pK_{a2}$ values for the amino acid residues are 7.7 and 9.3, respectively. This means that in the PAG there is a group with a $pK_a$ value of 9.3 that must be protonated for enzymic activity, and another with $pK_{a1}$ value of 7.7 that must be unprotonated for PAG activity. These values are consistent with the $pK_a$ values of cysteine and histidine, respectively. These values are about one unit higher than the $pK_a$ values of the cysteine and histidine; but the possibility of a shift in the $pK_a$ values (about one unit) of the amino acid residues of the native proteins is well known. However, the hypothesis, that cysteine and histidine could be implicated in the active site of the PAG, must be supported by other experimental approaches, such as the use of chemical modifiers.

![Fig. 1. Effect of pH on phosphate-activated glutaminase isolated from Ehrlich ascites tumour cells. Activity was assayed at different pH values as described in Section 2.](image1)

![Fig. 2. Kinetics of inactivation of purified tumour glutaminase by N-ethylmaleimide. At indicated times, PAG activity was assayed; for each NEM concentration, 0.1 mM (●), 0.25 mM (▲), 0.5 mM (●) and 0.75 mM (▼), the observed pseudo-first-order rate constant for inactivation, $k_{obs}$ was obtained.](image2)
3.2. Inactivation of PAG by N-ethylmaleimide

The sensitivity of Ehrlich PAG to inactivation by N-ethylmaleimide was determined in the isolated enzyme by incubation with 0.1, 0.25, 0.50 and 0.75 mM NEM concentrations. Fig. 2 displays the natural logarithms of the percent of remaining activities versus times of incubation. Under the experimental conditions, the respective pseudo-first-order rate constants for inactivation, $k_{\text{inact}}$, were 0.056, 0.132, 0.226 and 0.422 min$^{-1}$. The incubation of the enzyme with 0.75 mM NEM resulted in nearly complete inactivation of tumour PAG. Replotting the values of pseudo-first-order constants versus NEM concentrations, the value of the second-order rate constant for inactivation, $k_{\text{inact}}$, was obtained (Fig. 3); this value was 538 M$^{-1}$ min$^{-1}$. A double logarithmic plot of the pseudo-first-order constants versus NEM concentrations; the slope yields a reaction order of 0.96 with respect to NEM.

3.3. Inactivation of PAG by diethyl pyrocarbonate

Incubation of isolated PAG with DEPC in buffer pH 7.2 resulted in a time-dependent loss of PAG activity. At pH values between 5.5 and 8.0 DEPC selectively reacts with histidine, forming an N-dicarbethoxy histidyl derivative, which has a high absorbance at 242 nm. The spectrophotometric studies of purified PAG protein at 10 μM treated at pH 7.2 with DEPC 1.0 mM showed a maximum at 240 nm (Fig. 4). The absorption between 230 and 250 nm markedly increased in a time-dependent manner, while at 278 nm it did not decrease. The number of histidine residues modified by DEPC in isolated PAG is calculated to be two per each molecule of enzyme, using a molar extinction coefficient of 3200 M$^{-1}$ cm$^{-1}$. The DEPC is hydrolysed in aqueous solutions and the calculated half-life of the reagent is 17 min. The incubation time selected for the experiments was 10 min, less time than the half-life of the DEPC. Table 1 shows the inactivation of PAG at different concentrations of DEPC. These results described with isolated enzyme seem to confirm that, at least, one cysteine residue and one histidine residue are implicated in the active site of the tumour glutaminase.

3.4. Experiments with mitochondrial membrane-bound glutaminase

It is well known that the presence of protein ligands can protect the reaction of amino acid residues...
with group specific reagents. In the case of PAG, a simple way of testing this point would be to allow the protein to react with NEM in the presence of glutamate. Glutamate has been described as a competitive inhibitor for kidney type glutaminase [17]. For protection experiments it is necessary to separate the enzyme from the ligand in the determination of activity, since in this case the ligand is glutamate, and the glutaminase activity is determined by measuring the glutamate produced in the glutaminase reaction. This was possible using membrane-bound enzyme, which can be separated by washing the mitochondria after treatment with protective ligand, and successive centrifugation, as described in Section 2. In contrast, using purified enzyme the separation was not easily feasible without losing the enzyme activity, due to the instability of glutaminase.

In order to carry out the protection experiments using membrane-bound enzyme, it was, first of all, necessary to confirm both the inhibition by glutamate and the inactivation by NEM of mitochondria membrane-bound PAG, in a similar way as in isolated enzyme. Fig. 5 shows the double reciprocal plots of glutamate inhibition of membrane bound glutaminase at high (200 mM) and low (20 mM) phosphate concentrations. The inhibition of glutaminase by glutamate appears to be competitive at both, high and low phosphate concentrations, with apparent $K_i$ values of 23 and 1 mM, respectively. On the other hand, the membrane bound glutaminase was also inactivated by NEM. Results are very similar to those obtained with purified enzyme. The double logarithmic plot of the inactivation rate constant, $k_{obs}$, versus NEM concentration yields a reaction order of 0.96, which is compatible with one essential cysteine residue in the active enzyme (results not shown).

Under conditions which resulted in an 82% of PAG inactivation by NEM, the addition of 20 mM glutamate reduced the extent of inactivation to 58%; glutamine, the substrate of PAG reaction, afforded a lower but significant protection. The glutamate protection of the NEM alkylation was abolished when mitochondria were incubated in the presence of glutamate and 200 mM phosphate. Furthermore, glutamate also protected to a similar extent against PAG inactivation by other maleimides of different bulkiness and hydrophobic properties, namely: N-(1-pyrene)maleimide, and N-phenyl maleimide (data not shown). The concentrations of these two maleimides needed for completing PAG inactivation were considerably lower than the concentration of NEM, under the same experimental conditions.

The effect of glutamate concentration on the NEM inactivation half times is depicted in Fig. 6. A disso-

### Table 1

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<thead>
<tr>
<th>DEPC (mM)</th>
<th>Activity (%)</th>
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<tr>
<td>0</td>
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<tr>
<td>0.67</td>
<td>36</td>
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<tr>
<td>2.0</td>
<td>18.5</td>
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<td>3.3</td>
<td>12.2</td>
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<tr>
<td>6.7</td>
<td>10.7</td>
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The experiments were carried out as described in Section 2. The enzyme was preincubated with different concentrations of DEPC in buffer HEPES-KOH pH 7.2 for 10 min. Control incubations without DEPC were run in parallel.
The dissociation constant for glutamate at the binding site from which it protects against inactivation by NEM, was obtained by fitting the observed pseudo-first-order rate constants for inactivation, \( k_{\text{obs}} \), to the following equation [24]:

\[
k_{\text{obs}} = \frac{[\text{NEM}] (k_{1 \text{act}} K_1 + k'_{1 \text{act}} [\text{Glu}])}{(K_1 + [\text{Glu}])}
\]

Where \( K_1 \) is the dissociation constant for enzyme–glutamate complex and \( k_{1 \text{act}} \) and \( k'_{1 \text{act}} \) represent the rate constants for reaction of NEM with the free enzyme and the enzyme–glutamate complex, respectively. The fitted value of \( k'_{1 \text{act}} \) was zero, which favours the view of NEM only reacting with the free enzyme. On the other hand, the dissociation constant thus obtained for glutamate is 0.9 mM. The inhibition constant for glutamate determined from its competitive inhibition of the catalytic reaction at low phosphate was 1.0 mM. The agreement of these dissociation constants further reinforces that the glutamate protection results from binding to the active site. These data support the view of a reactive cysteine residue of glutaminase located at, or near, the active site.

4. Discussion

The main objective of pH studies is to identify the prototropic group implicated at the active site of the enzyme; thus, with some limitations, the \( pK_a \) values of prototropic amino acids can be determined by measuring the pH dependence of enzyme activity. The shape of the experimental curve (Fig. 1) matches the theoretical model of a diprotic enzyme. Taking into account this similarity and those values of pH at which the activity is the half activity in the optimum, the \( pK_{a1} \) and the \( pK_{a2} \) were calculated. These experimental values are consistent with the \( pK_a \) values of histidine and cysteine residues inside the protein [25]. Indeed, the reactivity of PAG with maleimides of different hydrophobic properties suggests that the active site should be located in a hydrophobic pocket; this fact could satisfactorily explain the \( pK_a \) experimental values about one unit higher than the theoretical values of \( pK_a \) for histidine and cysteine residues.

The chemical modifier N-ethylmaleimide is a sulfhydryl reagent widely used to characterise cysteine residues of proteins implicated in the catalysis [26]. With regard to glutaminases, only partial inhibition (40–60%) by 1.0 mM NEM was detected in pig renal mitochondrial and rat brain synaptosomal glutaminases [19,27]. In contrast, the rat liver enzyme was completely inactivated by NEM [17]. Much less is known for tumour glutaminase. Ehrlich ascites cell PAG can be fully inactivated by NEM treatment. The results of NEM alkylation studies in purified enzyme indicate that there is not a reversible complex formed prior to the inactivation process and also precludes any significant base-dependent decomposition of NEM [28]. The reaction order with regard to the thiol reagent was consistent with one essential residue being alkylated on the PAG protein. The second-order rate constant for inactivation, \( k_{1 \text{act}} \), deduced from the kinetic study indicates a very reactive residue, most likely a thiolate, being modified by NEM at pH 8.0 [29].

It is noteworthy that the studies carried out with membrane-bound enzyme in whole mitochondria confirm those obtained with purified enzyme, since NEM is a membrane permeant sulfhydryl reagent. The lack of sensitivity towards 1.0 mM NEM showed by the mitochondrial glutamine carrier in
these cells [30] clearly differentiates the two proteins. Thus, in both mitochondrial membrane-bound glutaminase and isolated enzyme the inhibition of glutamine hydrolysis by NEM seems to reflect the alkylation of an essential cysteine residue in the protein.

To gain further insights in the NEM-binding site, the effect of glutamate, a competitive inhibitor, and glutamine on the NEM inactivation of PAG were explored. Glutamine and glutamate protected against enzyme inactivation. Even though these experiments were carried out with the membrane-bound enzyme, it could be speculated that glutamate protection would also take place in purified enzyme. From kinetic studies, it is concluded that glutamate inhibition is competitive with glutamine and that the enzyme affinity for glutamate is increased at low phosphate concentrations (Fig. 5). Interestingly, the ability of glutamate to protect the PAG against inactivation by NEM can be reversed by phosphate. Moreover, the dissociation constants of the enzyme–glutamate complex, obtained from kinetic and protection studies were very close (1.0 and 0.9 mM, respectively). It therefore appears that the cysteine NEM-sensitive residue is located on the active site. However, it should be noted that definitive conclusions about the number of cysteines involved had to wait until knowledge of the stoichiometry of modification.

To our understanding, the existence of another essential amino acid for PAG activity, apart from cysteine, is so far unknown. The use of DEPC in the modification of enzymes has been reviewed [31]. DEPC has become a useful reagent for histidine modification in proteins, since at pH values ranging from 5.5 to 8.0 it reacts selectively with histidine residues [32]. The present studies confirm the sensitivity of purified glutaminase of Ehrlich cells to inactivation by DEPC. The specificity of the modification of essential histidine residues by DEPC was supported by the increase in the absorption of isolated enzyme between 230 and 240 nm, without any decrease in absorption near 278 nm after addition of DEPC. The increase near 240 nm is indicative of carbethoxylation of histidine residue, whereas the decrease at 278 nm is associated with O-acetylation or O-alkylation [33,34]. The present studies in isolated tumour glutaminase demonstrate, for the first time, the presence of essential histidine and cysteine residues at the active site of the enzyme.

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