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Microcalorimetric studies on the creatine kinase-catalyzed reaction in the presence of guanidine hydrochloride

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Abstract

This paper reports a thermodynamic method for the two-substrate enzyme-catalyzed reaction by a random sequential mechanism in the presence of a chemical denaturant. This is a convenient method to produce not only the apparent molar thermodynamic constants ($\Delta_r H_{m,a}$ and K_a) but also the standard thermodynamic properties of the reaction ($\Delta_r H_m^{\oplus}$, $\Delta_r G_m^{\oplus}$, $\Delta_r S_m^{\oplus}$). Microcalorimetry has been used to investigate thermodynamics of the reversible phosphoryl transfer from ATP to creatine catalyzed by rabbit muscle-type creatine kinase (MM-CK) at different concentrations of guanidine hydrochloride (GuHCl). From a thermodynamic viewpoint, this enzyme-catalyzed reaction follows a rapid-equilibrium, random mechanism, i.e. the chemical steps are slower than those for binding of reagents, and there is no obligatory order of binding or release. At 298.15 K, the standard enthalpy, Gibbs free energy, and entropy changes for the reaction at low concentrations of GuHCl were determined by this method to be 25.76 kJ mol^{-1}, 14.1 kJ mol^{-1}, and 38.9 J K^{-1} mol^{-1}, respectively, in agreement with those in the absence of GuHCl. The experimental results demonstrated the reliability of the above thermodynamic method, and indicated that inactivation of CK by low concentrations of GuHCl had no effect on the standard thermodynamic parameters for the CK-catalyzed reaction. A novel method for the determination of creatine kinase activity, the microcalorimetric assay for CK, was also proposed in this paper. The experimental results showed that GuHCl had a noticeable influence on the activity of CK.

Keywords: Creatine kinase; Guanidine hydrochloride; Inactivation; Microcalorimetry; Thermodynamics

1. Introduction

Creatine kinase (ATP: creatine phosphotransferase, CK, EC 2.7.2.3) is a key enzyme in cellular energy metabolism and plays an important role in the creatine–creatine phosphate energy shuttle during muscle contraction *in vivo* [1–9]. This enzyme catalyzes the reversible transfer of high-energy phosphate between ATP and phosphocreatine (PCr) in the presence of Mg^{2+}, during which an equimolar quantity of hydrogen ions are released:

$$\text{Creatine} + \text{MgATP}^{2-} \rightleftharpoons \text{Phosphocreatine}^{2-} + \text{MgADP}^{-} + \text{H}^{+} \quad (1)$$

Cytosolic creatine kinase from rabbit muscle (MM-CK) is a dimer which is composed of two identical 43 kDa polypeptide chains of known sequence [10]. The recently solved crystal structure of the isoenzyme at 2.35 Å resolution reveals that the dimeric interface

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of the enzyme is held together by only eight hydrogen bonds [11]. Guanidine hydrochloride (GuHCl) is a chemical denaturant often used in the investigations of protein folding [12]. Although many studies on the comparison of conformation and activity changes of creatine kinase denatured by GuHCl have been reported [13–19], little information on the thermodynamics of creatine kinase-catalyzed reaction in the presence of GuHCl is available at the present time.

Microcalorimetry is an important tool for the study of both thermodynamic and kinetic properties of biological macromolecules by virtue of its general applicability, high accuracy and precision, and developments in its use in recent years [20–22]. This method has many advantages over other conventional bioassay procedures, because there is no constraint on the solvent or the spectral, electrochemical or other properties of the substances involved in the reaction system. Measurements can be made directly without further addition of reagents, thus the progress of a reaction is not perturbed. Furthermore, microcalorimetry is a non-destructive technique and allows in situ biochemical analysis of the samples under investigation after the calorimetric curve of a reaction is measured. Owing to these advantages, microcalorimetry has been used extensively in the study of enzyme-catalyzed reactions during the past decade [23–29].

In this paper, the reversible phosphoryl transfer from ATP to creatine, catalyzed by rabbit MM-CK in the presence of GuHCl, has been investigated by microcalorimetry, based upon the microcalorimetric studies on the arginase [24,25], catalase [26], xanthine oxidase [27] and superoxide dismutase [28] catalyzed reactions. A thermodynamic method for the two-substrate enzyme-catalyzed reaction by a random sequential mechanism in the presence of a chemical denaturant is proposed. In order to test the validity of this method, we have applied microcalorimetry to the study on the thermodynamics of creatine kinase-catalyzed reaction in the presence of GuHCl. This phosphoryl transfer was a well-studied two-substrate reversible enzyme-catalyzed reaction followed the above type of mechanism [3]. The standard enthalpy, Gibbs free energy, and entropy changes for the reaction at low concentrations of GuHCl, calculated by the proposed method, were reported for the first time. The influence of GuHCl on both the standard thermodynamic properties of the CK reaction and the activity of the enzyme was also discussed.

2. Theory and method

For an enzyme-catalyzed reaction with a rapid equilibrium, random, bimolecular, bimolecular mechanism in the presence of a chemical denaturant, its apparent equilibrium constant, K_a, may be written as

$$K_a = \frac{x^2}{(C_{A,0} - x)(C_{B,0} - x)} \tag{2}$$

where $C_{A,0}$ and $C_{B,0}$ are the initial concentrations of the substrates A and B, respectively; and x is the concentration of the products C and D, which can be determined by the following formula:

$$x = \frac{Q_{a,\infty}}{V_T \Delta_r H_{m,a}} \tag{3}$$

Here, $Q_{a,\infty}$ is the total apparent heat effect of an enzymatic reaction which can be calculated by the integration type of Tian equation [21,24,27] from the measured calorimetric curves, V_T the total volume of the reacting system (in this paper, V_T=6.00 ml), and $\Delta_r H_{m,a}$ the apparent molar enthalpy change of the reaction.

Substituting Eq. (3) in Eq. (2), we obtain

$$K_a = \frac{Q_{a,\infty}^2}{(C_{A,0}\Delta_r H_{m,a} V_T - Q_{a,\infty})(C_{B,0}\Delta_r H_{m,a} V_T - Q_{a,\infty})} \tag{4}$$

This novel thermodynamic model can be used to perform a nonlinear least-squares analysis of $Q_{a,\infty}$ as an explicit function of $C_{A,0}$ and $C_{B,0}$. Then two unknown thermodynamic parameters, K_a and $\Delta_r H_{m,a}$, can be obtained by fitting the experimental data ($C_{A,0}$, $C_{B,0}$, and $Q_{a,\infty}$) to the model using the Origin software provided by Microcal Software (version: 5.0).

For the CK-catalyzed reversible phosphoryl transfer from ATP to creatine in the presence of a chemical denaturant and at a fixed pH, its standard equilibrium constant is

$$K^{\oplus} = K_a \frac{C_{H^+}}{C^{\oplus}} \tag{5}$$

where C_{H^+} is the concentration of H^+ in the reacting system, $C^{\oplus}$ the standard concentration of H^+ (here, $C_{H^+}=10^{-9}$ mol dm^{-3}, and $C^{\oplus}=10^{-7}$ mol dm^{-3}).

Therefore, the standard molar enthalpy change, $\Delta_r H_m^{\oplus}$, the standard molar Gibbs free energy change, $\Delta_r G_m^{\oplus}$, and the standard molar entropy change, $\Delta_r S_m^{\oplus}$, for the CK-catalyzed reaction in the presence of a chemical denaturant can be calculated by the following relationships, respectively:

$$\Delta_r H_m^{\oplus} = \Delta_r H_{m,a} - \Delta_r H_{m,P} \quad (6)$$

$$\Delta_r G_m^{\oplus} = -RT \ln K^{\oplus} \quad (7)$$

$$\Delta_r S_m^{\oplus} = \frac{\Delta_r H_m^{\oplus} - \Delta_r G_m^{\oplus}}{T} \quad (8)$$

Here, $\Delta_r H_{m,P}$ is the molar enthalpy change for a coupled reaction, the protonation of the sodium salt of the glycine buffer, concurrent with the production of hydrogen ions by Reaction 1 (Eq. (1)):

$$NH_2CH_2COO^- + H^+ \rightleftharpoons^{+} NH_3CH_2COO^- \quad (\text{fast}) \quad (9)$$

Using microcalorimetry, $Q_{a,\infty,P}$, the total apparent heat effect of mixing a known concentration of hydrochloric acid solution with an excess of 0.1 mol dm^{-3} glycine–NaOH buffer (pH=9.0), can be measured. The value of $\Delta_r H_{m,P}$ can then be calculated from the following equation:

$$\Delta_r H_{m,p} = \frac{Q_{a,\infty,P} - Q_d}{V_T C_{HCl,0}} \quad (10)$$

where Q_d and $C_{HCl,0}$ are the dilution heat and initial concentration of hydrochloric acid, respectively.

Obviously, the method proposed herein is also suitable for studying the two-substrate enzyme-catalyzed reaction by a random sequential mechanism in the absence of a chemical denaturant.

3. Experimental

3.1. Reagents

Rabbit MM-CK was prepared and further purified as described previously [2]. The $A_{1\,cm}^{1\%}$ value of 8.8 was used for protein concentration measurement [18]. The concentration of MM-CK solution was 6.26 mg cm^{-3} and the specific activity of this enzyme determined by using the indicator colorimetry method developed in this laboratory [15,30] to be 130 U mg^{-1}. Ultra pure GuHCl was purchased from ICN Biomedicals, USA. The CK–GuHCl mixed solution at each denaturant concentration was prepared by incubation of the enzyme in buffer with GuHCl at different concentrations at 4°C overnight. Creatine, glycine, and the disodium salt of ATP were Sigma products with purity ≥98%. Magnesium acetate ($MgAc_2$) and other chemicals used were domestic and of A.R. grade. All reagent solutions were prepared in 0.1 mol dm^{-3} glycine–NaOH buffer (pH=9.0). As the ATP solution undergoes slow hydrolysis, it was freshly prepared on each occasion.

3.2. Instrumentation

The heats of the reversible phosphoryl transfer from ATP to creatine catalyzed by MM-CK at different concentrations of GuHCl were determined at 298.15 K (25.00°C) using a LKB-2107 batch microcalorimeter equipped with a microtitrator unit from Sweden, which consists of a micro-batch reactor with a heat-conduction isothermal calorimeter. The instrument contains twin calorimeter vessels, one of which is the reaction vessel and the other a reference vessel, each vessel being divided by a partition wall into two compartments [24,31]. The enzyme–GuHCl mixed solution and the substrate solution, namely the creatine–ATP–$MgAc_2$–GuHCl mixture, had already separately diluted to the required concentrations with buffer before calorimetric experiments. The pH value of the substrate solution was adjusted to 9.0 by adding 0.2 mol dm^{-3} NaOH solution. Compartment I of reaction vessel contained 2.00 cm^3 of the CK–GuHCl mixed solution, and compartment II of reaction vessel contained 4.00 cm^3 of the substrate solution, in which the concentration of GuHCl was the same as that in compartment I. In order to avoid the influence of the heat effects of diluting, mixing etc. on the results of the measurement, the contents and quantities in both vessels were as close as possible except that CK was not added to the reference vessel. After thermal equilibrium of the microcalorimetry system and a steady baseline on the recorder had been obtained, the reaction run was initiated by starting rotation of the calorimeter 360° clockwise and then anti-clockwise, in order to mix the enzyme-denaturant and substrate solutions thoroughly. The output signal was recorded in the form of a calorimetric curve by means of the LKB-2210 dual-pen integration recorder. After the

calorimetric experiment on the phosphorylation, the pH value of the residual solution taken from the reaction vessel was almost the same as that from the reference vessel. Under the experimental conditions used, the heat effect of the dilution of CK was negligible and could be ignored.

It should be pointed out that the thermodynamic method proposed herein is also adapted to the contemporary isothermal titration calorimeters (ITC), such as a MicroCal Omega microcalorimeter from USA [23]. The heats of the phosphoryl transfer from ATP to creatine catalyzed by MM-CK at different concentrations of GuHCl will be conveniently measured with ITC by injecting the CK–GuHCl mixed solution into the reaction vessel filled with the substrate solution.

4. Results

4.1. Thermodynamic properties of CK-catalyzed reaction in the presence of GuHCl

Fig. 1 shows six measured calorimetric curves for the reversible phosphoryl transfer from ATP to creatine catalyzed by rabbit MM-CK at different concentrations of guanidine hydrochloride. It can be seen from Fig. 1 that the measured calorimetric curves for these CK reactions show an exothermic peak, indicating the apparent molar enthalpy changes for these reactions in the GuHCl concentration range 0–0.0300 mol dm^{-3} were negative under the conditions used. At concentrations of GuHCl higher than 0.500 mol dm^{-3}, the heat effect for the CK-catalyzed reaction was nearly zero, therefore, both the standard equilibrium constant and the standard molar enthalpy change for the reaction were zero.

Table 1 summarizes the thermodynamic data for the MM-CK-catalyzed reactions at different concentrations of guanidine hydrochloride and at 298.15 K, in which the apparent thermodynamic parameters for the CK reaction in the presence of GuHCl, K_a and $\Delta_r H_{m,a}$, are obtained by fitting the experimental data, the hollow circles in Fig. 2 (in part), with Eq. (4). Then their corresponding standard thermodynamic properties, $K^{\oplus}$, $\Delta_r H_m^{\oplus}$, $\Delta_r G_m^{\oplus}$, and $\Delta_r S_m^{\oplus}$, are calculated by Eqs. (5)–(8). The solid line in Fig. 2 is the total apparent heat effect for the CK reaction in the presence of 0.200 mol dm^{-3} GuHCl predicted by the parameters in Table 1. Table 2 lists the molar reaction enthalpy for the concurrent protonation of sodium salt of glycine at 298.15 K, in which the salt concentration greatly exceeds that of hydrochloric acid. As we can

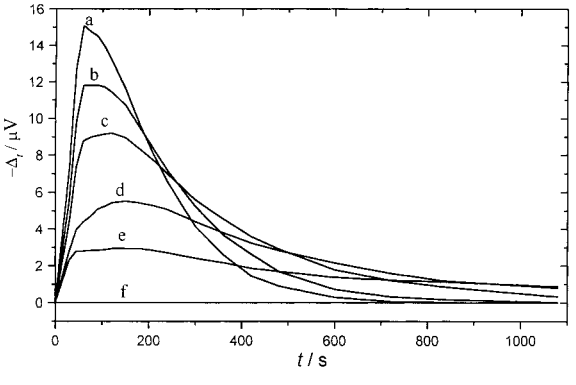

Fig. 1. Calorimetric curves of the reversible phosphoryl transfer from ATP to creatine catalyzed by MM-CK at different concentrations of GuHCl. The experimental conditions were T=298.15 K, pH=9.0, and the initial concentration of enzyme was 5.22×10^{-2} mg cm^{-3}, $C_{Cr,0}=7.18\times10^{-3}$ mol dm^{-3}, $C_{ATP,0}=1.41\times10^{-3}$ mol dm^{-3}, and $C_{MgAc_2,0}=1.72\times10^{-3}$ mol dm^{-3}. The initial concentrations of GuHCl are (a) 0, (b) 0.0300, (c) 0.100, (d) 0.200, (e) 0.300, and (f) 0.500 mol dm^{-3}, respectively.

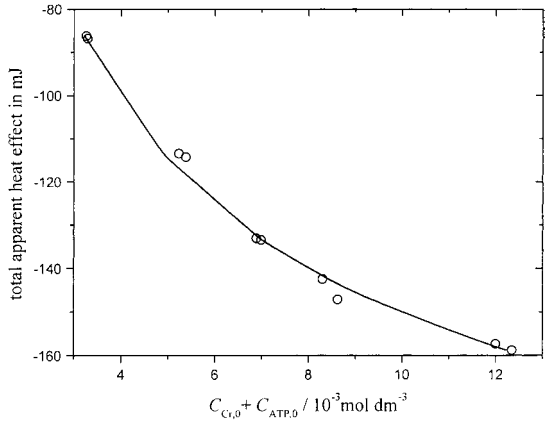

Fig. 2. Plot of the total apparent heat effect for the reversible phosphoryl transfer from ATP to creatine catalyzed by MM-CK against substrate concentration in the presence of 0.200 mol dm^{-3} guanidine hydrochloride. The experimental conditions were T=298.15 K, pH=9.0, and the initial concentration of enzyme was 5.22×10^{-2} mg cm^{-3}. The hollow circles are the experimental data, and the solid line is the theoretical curve predicted by the thermodynamic model (Eq. (4)) and the parameters in Table 1.

Table 1
Standard thermodynamic properties for the reversible phosphoryl transfer from ATP to creatine catalyzed by MM-CK at different concentrations of GuHCl and at 298.15 K[a]

$C_{GuHCl,0}$ (mol dm^{-3})	K_a	$-\Delta_r H_{m,a}$ (kJ mol^{-1})	$K^{\oplus}\times10^3$	$\Delta_r H_m^{\oplus}$ (kJ mol^{-1})	$\Delta_r G_m^{\oplus}$ (kJ mol^{-1})	$\Delta_r S_m^{\oplus}$ (kJ mol^{-1})
0	0.333±0.052	24.55±0.81	3.33[b]	26.02[b]	14.1[b]	40.0[b]
0.0100	0.345±0.048	25.09±0.78	3.45	25.48	14.1	38.2
0.0300	0.340±0.056	24.88±0.65	3.40	25.69	14.1	38.9
0.0500	0.336±0.039	24.98±0.66	3.36	25.59	14.1	38.5
0.100	0.350±0.057	25.20±0.74	3.50	25.37	14.0	37.6
0.200	0.326±0.050	24.76±0.80	3.26	25.81	14.2	38.9
0.300	0.314±0.042	23.97±0.68	3.14	26.60	14.3	41.3
0.500	0	0	0[b]	0[b]	∞[b]	$-\infty$[b]
1.00	0	0	0[b]	0[b]	∞[b]	$-\infty$[b]
Average values			3.35	25.76	14.1	38.9
Standard errors			0.13	0.44	0.1	1.3

[a] The initial concentration of enzyme was 5.22×10^{-2} mg cm^{-3}, and $C_{ATP,0}/C_{MgAc_2,0} = 1.22$.
[b] The values are excluded from the averages and discarded.

see from Table 1, these CK-catalyzed reactions at low concentrations of GuHCl are really endothermic although their apparent molar enthalpy changes were negative. As can also be seen in Table 1, the standard relative errors for these thermodynamic data, K_a, $\Delta_r H_{m,a}$, $K^{\oplus}$, $\Delta_r H_m^{\oplus}$, $\Delta_r G_m^{\oplus}$, and $\Delta_r S_m^{\oplus}$, are small, and these thermodynamic parameters are in agreement with those in the absence of GuHCl. This verifies the validity of the thermodynamic method used, and indicates that inactivation of CK by low concentrations of GuHCl has no effect on the standard thermodynamic parameters for the CK-catalyzed reaction. The experimental results also mean that from a thermodynamic viewpoint, this enzyme-catalyzed reaction follows a rapid-equilibrium, random mechanism even in the presence of GuHCl, i.e. the chemical steps are slower than those for binding of reagents, and there is no obligatory order of binding or release. It can be found from Fig. 2 that the model reported here yielded a curve that adjusted fairly to the experimental data as indicated by both the variance of the fit and the χ-statistics for the model (χ^2 for these reactions ranged from 0.1 to 0.5). Therefore, the appropriateness of the method is also tested statistically. From Table 2, it can be seen that the measured value of $\Delta_r H_{m,P}$ shows better reproducibility.

4.2. Influence of GuHCl on CK activity

In order to determine the activity of CK, the initial concentrations of two substrates, $C_{Cr,0}$ and $C_{ATP,0}$, must be greatly exceed $K_{m,Cr}$ and $K_{m,ATP}$, e.g., $10K_{m,Cr}$ and $10K_{m,ATP}$, respectively. Under this condition, the initial rate of heat evolution, P_0, is proportional to the

Table 2
Molar reaction enthalpy for the concurrent protonation of sodium salt of glycine at 298.15 K[a]

No.	$C_{HCl,0}\times10^4$ (mol dm^{-3})	$-Q_{a,\infty}$ (mJ)	$-Q_d$ (mJ)	$-\Delta_r H_{m,P}$ (kJ mol^{-1})
1	0.2991	9.1307	0.04390	50.63
2	0.7478	22.819	0.1098	50.62
3	1.496	45.344	0.2195	50.29
4	2.243	67.861	0.3293	50.17
5	2.991	91.656	0.4390	50.83
6	5.982	183.54	0.8780	50.89
Average value				50.57
Standard error				0.29

[a] The initial concentration of sodium salt of glycine was 1.2×10^{-2} mol dm^{-3}.

Table 3
MM-CK activities at different GuHCl concentrations at 298.15 K[a]

$C_{GuHCl,0}$ (mol dm^{-3})	P_0 (mJ s^{-1})	SA (U mg^{-1})	Remaining activity (%)
0	−1.433	111.8	100
0.0100	−1.392	106.3	95.1
0.0300	−1.130	87.01	77.8
0.0500	−1.057	81.06	72.5
0.100	−0.8927	67.86	60.7
0.200	−0.5317	41.14	36.8
0.300	−0.2714	21.69	19.4
0.500	0	0	0
1.00	0	0	0

[a] Concentrations in cell: CK, 5.22×10^{-2} mg cm^{-3}; ATP, 2.836×10^{-2} mol dm^{-3}; creatine, 4.376×10^{-2} mol dm^{-3}; $MgAc_2$, 3.516×10^{-2} mol dm^{-3}.

concentration of the enzyme, and the specific activity of the enzyme (SA) can be calculated by

$$\mathrm{SA} = \frac{P_0}{\Delta_r H_{m,a}[E]_0 V_T} \tag{11}$$

This is a novel method for the determination of creatine kinase activity, called the microcalorimetric assay for CK. The results of the measurement of MM-CK activities by this method at different GuHCl concentrations are given in Table 3. It can be seen from Table 3 that the value of SA for CK decreases with increase in the concentration of GuHCl. When the concentration of GuHCl exceeds 0.500 mol dm^{-3}, the remaining enzyme activity is almost zero. These analysis results show that GuHCl has a noticeable influence on the activity of CK.

As we can also see from Table 3, the specific activity of CK in the absence of GuHCl measured by the microcalorimetric assay (111.8 U mg^{-1}) is in agreement with that determined by the indicator colorimetry method (130 U mg^{-1}) [14,30] at the same experimental conditions. It shows that the microcalorimetric assay for CK proposed in this paper is feasible.

5. Discussion

5.1. On the standard state of hydrogen ions

In order to be able to compare different reactions of a particular substance, a common reference state must be defined, which in the field of thermodynamics is termed the standard state of the substance [32]. The primary standard state of hydrogen ions is unit activity (1 mol dm^{-3}, pH=0.0). However, this is not relevant to normal biological conditions, and so for biological systems a standard state for hydrogen ion concentration corresponding to pH=7.0 is used (a concentration of 10^{-7} mol dm^{-3}) [32]. The standard thermodynamic parameters of a biochemical reaction will vary with the standard state chosen. We have adopted pH=7.0 as the standard state for hydrogen ions in this paper, and label the corresponding standard functions as $K^{\oplus}$, $\Delta_r H_m^{\oplus}$, $\Delta_r G_m^{\oplus}$, and $\Delta_r S_m^{\oplus}$.

5.2. Advantages of the microcalorimetric method

In this paper, the microcalorimetric method was employed to study the thermodynamics of the CK-catalyzed reaction in the presence of GuHCl. Theoretically, this method can be used to calculate a complete set of standard thermodynamic parameters for a random, two-substrate enzyme-catalyzed reaction in the presence of a denaturant with the calorimetric data from only two independent experiments.

Compared with traditional non-calorimetric methods [32], the microcalorimetric method has two advantages. The first advantage is that it measures both the apparent reaction enthalpy and the apparent equilibrium constant directly and accurately, requiring fewer experiments. The second advantage is that the thermodynamic data need not be determined at different temperatures. For the traditional non-calorimetric methods, such as NMR, and spectrophotometric and fluorometric assays, the apparent equilibrium constants must be measured at a minimum of four different temperatures, in order to use the van't Hoff plot to determine the standard reaction enthalpies. Limitations of non-calorimetric methods include a requirement that the reaction enthalpy be temperature independent, and a lower degree of accuracy [32]. Consequently, the microcalorimetric method is particularly suitable for studying the thermodynamics of random, two-substrate enzyme-catalyzed reactions in the presence of a denaturant where the reaction enthalpy is temperature dependent. For these reasons, microcalorimetry is regarded as one of the most rigorous methods for the accurate measurement of

thermodynamic properties of many types of enzyme-catalyzed reactions.

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