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THE ACTIVITY OF PAPAIN IN THE CRYSTALLINE STATE*

L. A. Æ. SLUYTERMAN AND M. J. M. DE GRAAF
Philips Research Laboratories, N.V. Philips' Gloeilampenfabrieken, Eindhoven (The Netherlands)

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SUMMARY

The rate of conversion of dissolved substrate by a suspension of enzyme crystals is governed by the rate of diffusion and the reaction rate of the substrate inside the crystal. If the crystal is thin enough the diffusion is not rate limiting. This critical size, d_c, equals $\sqrt{K_m D_i/k_{cat}[E]}$, where D_i is the diffusion rate of the substrate, and $[E]$ the enzyme concentration inside the crystal. The value of d_c increases if either a substrate with a high value for K_m/k_{cat} (*i.e.*, a poor substrate) or crystals having low $[E]$ values (mixed crystals of mainly irreversibly inhibited enzyme and a small quantity of active enzyme, *etc.*) are used.

These principles were applied to two crystal modifications of papain, *A* and *C*. As a poor substrate acetylglycine ethyl ester was used; benzoylarginine ethyl ester was used in conjunction with mixed crystals of 98% papain inactivated with chloroacetamide and 2% active papain. With both substrates and both crystal modifications complete activity was observed when the effect of the suspension medium (20% Na_2SO_4) upon the reaction rate was taken into account. This latter effect was evaluated with cross-linked papain crystals insoluble in both water and 20% Na_2SO_4.

Data from the literature concerning three crystalline hydrolytic enzymes are discussed in terms of the above and other equations involving D_i, as derived in the appendix. It is concluded that chymotrypsin and ribonuclease-S crystals may, like papain, be completely active.

A convenient method of preparing mercuri-papain and *S*-carboxyamidomethylene papain is described.

INTRODUCTION

In studies concerning the relation between structure and function of enzymes, an essential problem arises. The most detailed knowledge of the structure of enzymes

Abbreviations: AGEE, acetylglycine ethyl ester; BAEE, benzoylarginine ethyl ester.

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is obtained from X-ray analytical data of crystals of the enzyme, whereas functional properties are generally studied in dilute solutions of the enzyme. It is not known a priori whether or not the structure of an enzyme is the same in solution and in the solid state. One way of finding out is to determine whether or not the activity of the enzyme is the same in both states. Up to now a certain number of such studies have been made, with varying results. On the one hand reasonably high activity was reported in crystalline ribonuclease-S (ref. 1) (5–25%) and in chymotrypsin[2] (20%). On the other hand very low reactivity was observed in crystalline carboxypeptidase[3] (0.3%) and liver alcohol dehydrogenase[4] (0.1%). In the latter enzyme, the combination rate of apoenzyme with coenzyme was measured. An intermediate case is the reaction of ferrimyoglobin with azide, the rate of which in the crystalline protein is 5% of the rate in solution[5]. The reaction of azide with crystalline ferrihemoglobin is a biphasic process, the steps of which have rates equal to 40% and 5% of the rates of the soluble protein[6].

These examples demonstrate that there is no general rule concerning enzyme reactivity in the crystalline state and that every enzyme and possibly every crystal modification of each enzyme, must be examined separately in this respect.

The present paper reports the results of investigations on the reactivity of crystalline papain of two crystal modifications, *A* and *C* (ref. 7).

Considerations on the diffusion limitation

The rate of conversion of a dissolved substrate by a suspension of enzyme crystals is governed by the rate of diffusion of the substrate into and inside the crystal and the reaction rate inside the crystal. In comparatively large crystals, substrate molecules will be converted into products soon after entering the crystal and before they are able to penetrate into its centre. Hence only the enzyme layer near the surface will take part in the reaction. This results in an apparently low activity per mole of enzyme. If the size of the crystals is decreased to such an extent that the reacting layers of the different crystal faces sufficiently overlap, diffusion will no longer be a limiting factor. It can be calculated (see APPENDIX) that this critical size, d_c, for steady-state conditions equals:

$$d_c = \sqrt{\frac{K_m \, D_i}{k_{cat} \, [E]}} \qquad (1)$$

In this equation d_c denotes the smallest diameter of the crystal, K_m and k_{cat} the Michaelis–Menten parameters of the substrate, D_i the rate of diffusion of the substrate inside the crystal and $[E]$ the free enzyme concentration in units of moles of enzyme per volume of liquid inside the crystal (*cf.* ref. 1). Since most protein crystals contain about 50% of liquid one can, as a first approximation, take D_i to be equal to the rate of diffusion in free solvent corrected for certain effects discussed in the appendix.

Equation (1) shows that the critical size can be increased by adjusting two factors: (a) make K_m/k_{cat} high, *i.e.*, use a poor substrate, (b) make $[E]$ low. Although it is obviously impossible to decrease the number of protein molecules per unit volume of crystal, $[E]$ can effectively be lowered by using mixed crystals of irreversibly inhibited enzyme and active enzyme or by using pH values removed from the optimum pH value of the enzyme, *etc.*

The use of high substrate concentrations, $[S] \gg K_m$, also lowers the effective free-enzyme concentration owing to the formation of ES complex. However, it may not always be experimentally feasible to make $[S]$ high enough to increase d_c markedly.

There is an additional advantage in using procedure (a) or (b) (or both at the same time if need be). In many cases, protein crystals will not be completely insoluble in the suspension medium. Procedures (a) and (b) require the use of fairly large aliquots of enzyme in order to obtain readily measurable rates of substrate conversion. This makes the ratio of suspended to dissolved enzyme high and hence improves the distinction between the effects of dissolved and of suspended enzyme. Both procedures have been used in the present investigation.

Elimination of solvent effects

In general the medium in which enzyme crystals are sufficiently insoluble differs greatly from the medium in which the enzyme is soluble and in which kinetic measurements are carried out. For instance, strong salt solutions are used for the activity assay of the crystal suspension and dilute salt solutions for the assay of dissolved enzyme. The effect of the medium on the kinetic properties of the enzyme may be considerable. This presents a problem because, on the one hand, the crystals are not stable at the conditions where the enzyme is soluble and, on the other hand, the enzyme is not sufficiently soluble in the medium in which the crystals are stable, thus making the proper evaluation of the medium effect difficult.

This problem can be solved by using cross-linked crystals which are insoluble in all media. Although the activity of cross-linked crystals may be lower than the activity of ordinary crystals, this does not matter since only the ratio of activities in the two media is of interest.

In the present investigation the cross-linking agent glutaraldehyde was used.

MATERIALS AND METHODS

Equipment

Progress curves of enzymic ester hydrolysis were recorded on the pH-stat combination of Radiometer (Copenhagen, Denmark) consisting of the pH meter (type TTT 1), titrigraph (type SBR2) and syringe burette (SBU1) or, later on, autoburette (type ABU1). At full-scale deflection of the recorder, 0.5 ml 0.1 M NaOH (syringe burette) or 0.25 ml 0.2 M NaOH (autoburette) was added to the reaction vessel of 12 ml.

Reaction conditions

The reaction mixtures contained 0.3 M KCl, 5 mM cysteine and 1 mM EDTA for dissolved papain; for the crystal suspensions the KCl solution was replaced by 20 g Na_2SO_4 in 100 ml total volume and the same cysteine and EDTA concentrations. The pH was 6.0, *i.e.*, the optimum pH of papain action, the temperature was 25° thermostated to within $\pm$ 0.05°, and the reaction volume was 10 ml. For kinetic measurements mercuri-papain was preferred, because this is most stable and is immediately and completely activated under the conditions given above. The Michaelis–Menten parameters of acetylglycine ethyl ester (AGEE) were determined at six substrate concentrations ranging from 0.05 M to 0.3 M, two determinations at each concentration. The data were plotted as $1/v$ *versus* $1/[S]$.

Preparation of papain

Papain was prepared according to the method of KIMMEL AND SMITH[8], with modifications in the first two steps.

Step 1. Papaya latex (180 g) was powdered, mixed with 100 g celite, 150 g washed quartz sand and 300 ml of 0.04 M cysteine (7.0 g cysteine hydrochloride *plus* 2.16 g NaOH in 1 l) and ground in an electric mortar for 1.5 h. The suspension was filtered on two Whatman No. 1 filters on top of each other in an 18-cm Buchner funnel, with suction. The almost dry filter cake was washed three times with a total volume of 700 ml of the cysteine solution.

Step 2. The precipitate of this step was removed by centrifugation instead of filtration. Both steps were carried out at room temperature.

For Step 3 $(NH_4)_2SO_4$ of special enzyme grade (Mann Research Laboratories Inc., New York) was utilized. The final yield was 1.4–2.3 g papain, usually in the form of a 6–7% papain-containing gel-like suspension.

Crystallization of modification A

To a certain amount of papain gel half its volume of methanol was added. Insoluble material was removed by centrifugation. Sufficient methanol was added to make the final solution 60% methanol (by vol.). The pH was 6–7. After a few days at room temperature or in the refrigerator, crystallization was complete as viewed with a dark-field microscope.

Crystallization of modification C

2 g of papain gel are mixed with 1 ml methanol. The turbid solution is clarified by centrifugation and, if necessary, by filtration through a millipore filter. 3 ml methanol and 6 ml 66% methanol (by vol.) containing 0.20 M glycine buffer, pH 9.6 or pH 10.1, are added. The mixture is diluted with 66% methanolic 0.10 M glycine buffer to adjust the final papain concentration. Readjustment of the pH with strong acid or base in the presence of papain should be avoided. Small crystals of about 1 μ thick were obtained from 1% papain at pH 9.6, larger crystals, up to 6 μ thick, were obtained from 0.3% papain at pH 10. In general, mixtures of single crystals and crystals grown in small rosettes were obtained. For complete crystallization of modification *C* in 1 or 2 days the mixtures were subjected to a periodic change of temperature. The temperature was increased from 0° to 25° in 5 min, kept at 25° for another 5 min and reduced to 0° in the course of 1 h, resulting in 20 cycles a day. Moreover, the solutions were gently swirled by a horizontal rotational motion in a circle of 4 cm diameter at a rate of 2 rev./sec.

Preparation of mercuri-papain

Papain crystals of modification *A* or *C* in, for example, 20 ml 66% methanol were centrifuged down, the supernatant was removed and the crystals were resuspended in 20 ml of 15% Na_2SO_4 in water containing 5 mM cysteine and 1 mM EDTA (pH 6.0) for activation. This sudden change of medium does not harm the crystals (B. G. WOLTHERS, private communication). The crystals were centrifuged again and the supernatant was replaced by 20% Na_2SO_4 containing the same concentrations of cysteine and EDTA for complete activation. The crystals were centrifuged and resus-

pended in 20 ml Na_2SO_4 containing 1 mM $HgCl_2$. The resulting crystals were washed twice with 20 ml 20% Na_2SO_4.

This procedure of preparing mercuri-papain has several advantages over a method using gel filtration: the activation is more complete, larger batches can be handled and no dilution occurs on the way.

Preparation of carboxyamidomethylene papain

Papain crystals are activated as described above and are suspended in 20% Na_2SO_4 containing 0.05 M acetate (pH 6.0), 1 mM EDTA and 0.05 M chloroacetamide. After 30–60 min at room temperature, the crystals are washed twice with 20% Na_2SO_4.

The crystals are dissolved in water at pH 5 to make a 0.5% protein solution and checked for residual activity. If necessary a small amount of mercuri-papain is added to adjust the final activity to about 2% of the activity of the initial preparation. The mixture is precipitated by the addition of NaCl up to 0.2 M. After storage at 5° for one night, the precipitate is centrifuged. The resulting gel is subjected to one of the crystallization procedures described above.

Preparation of cross-linked crystals (cf. ref. 3)

Crystals of mercuri-papain or of mixed crystals are suspended in 20% Na_2SO_4 containing 1% glutaraldehyde and 0.05 M phosphate (pH 7.5) and slowly shaken at room temperature. After 15 min the mixture is 10-fold diluted with 0.02 M acetate pH 6. The crystals are extensively washed with water and, if necessary, resuspended in 20% Na_2SO_4.

RESULTS

Activity towards a poor substrate

AGEE proved to be a suitable substrate. It was found to have a K_m of 0.45 M. The k_{cat} was found to be about 10 times lower than the k_{cat} of benzoylarginine ethyl ester (BAEE). Since BAEE has a K_m of 0.018 M, the K_m/k_{cat} value of AGEE is 250 times lower than that of BAEE. The relative k_{cat} value of AGEE is only an approximate one, since the activity appeared to be somewhat dependant upon enzyme concentration, as will be reported below.

For activity determination of the crystalline state, 0.1-, 0.2-, 0.3- and 0.4-ml aliquots of a stock suspension of papain crystals (about 20 mg protein per ml) in 20% Na_2SO_4 were added to 10 ml of 20% Na_2SO_4 which contained 0.05 M AGEE as substrate and 5 mM cysteine and 1 mM EDTA as activators, in the reaction vessel of the pH-stat. For comparison with the reactivity in the dissolved state, aliquots of the same papain suspension were added to 10 ml 0.3 M KCl, everything else being the same. Under these standard conditions, the papain crystals dissolved. The resulting activities, in terms of base consumption per min, are shown in Fig. 1. It is quite evident that the apparent activity of the crystals in high sulphate solution is 5 times higher than the same amount of papain dissolved in 0.3 M KCl.

Another point of interest apparent on closer inspection of Fig. 1 is that there is no proportionality between the amount of papain and activity, neither in solution nor in suspension. In both cases the activity in proceeding from the 0.1-ml aliquot of papain to the 0.4-ml aliquot increases only 3.4 times instead of 4.0 times (after cor-

rection for the increase in reaction volume caused by the papain aliquots). The reason for this effect is not clear. Whereas in solution such an effect might be caused by increasing enzyme aggregation with increasing concentration, this explanation does not apply to a suspension of crystals. In the latter case, the degree of association of the enzyme inside the crystals cannot change on the addition of more crystals to the suspension. However this may be, the main interest is the ratio of activities of crystalline and dissolved enzyme, which is the same at all "concentrations" of papain. In the experiment of Fig. 1, the mean value of this ratio was 5.4. In three other similar experiments, ratios of 4.9, 5.3, and 4.9 were found. The mean value was 5.1.

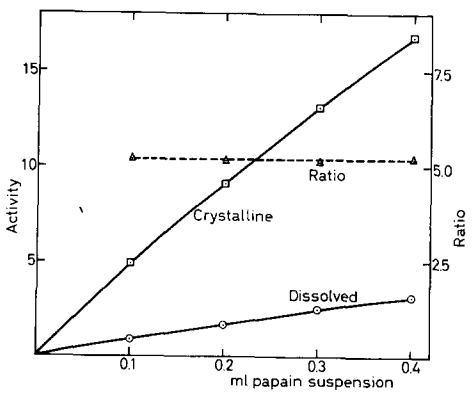

Fig. 1. Activity of dissolved and of suspended crystalline papain modification *A* as a function of the quantity of papain, with AGEE as substrate.

In order to determine the extent of dissolution of the papain crystals in the suspension medium, 0.4 ml papain suspension was introduced into 10 ml reaction mixture lacking substrate. After 5 min of stirring, the crystals were removed by centrifugation. The supernatant was again introduced into the reaction vessel of the pH stat, substrate was added and the hydrolysis rate was measured. It was found to be only 8% of the activity observed in the parent experiment in which the crystals were not removed by centrifugation. Hence only a fraction of the observed activity was due to dissolved papain.

The effect of Na_2SO_4 upon the activity was evaluated by repeating the above-mentioned experiments with cross-linked papain crystals insoluble both in 20% Na_2SO_4 and in 0.3 M KCl. The ratio of activities in these media proved to be 3.8. Hence the activity of the papain crystals towards AGEE, corrected for the sulphate effect, amounts to $5.1/3.8 \times 100\% = 134\%$.

In 10% Na_2SO_4 mercuri-papain was sufficiently soluble for an activity determination towards AGEE. This permitted a check on the use of cross-linked crystals for the evaluation of the medium effect. The ratio of the activities of dissolved mercuri-papain towards 0.05 M AGEE in 10% Na_2SO_4 and in 0.3 M KCl was 2.00. With a suspension of cross-linked crystals this ratio was 1.98. Hence the use of cross-linked crystals for evaluating medium effects was justified.

Similar experiments were carried out with crystals of modification *C* with similar results. The same deviation from linearity as shown in Fig. 1 was observed, and there was a similar low solubility of the crystals in the suspension medium. On

the other hand, the ratio of the activities of crystalline and dissolved enzyme was 3.7. After correction for the medium effect, this corresponds to 97% activity of papain crystals of modification *C*.

Activity towards a good substrate

The activity of crystalline papain towards 0.02 M BAEE was examined with mixed crystals of 2% mercuri-papain and 98% *S*-carboxyamidomethylene papain. The results were quite similar to those for AGEE as far as the dependance on papain "concentration" and the low percentage of activity which dissolved* in 20% Na_2SO_4 are concerned. With both crystal modifications, the ratio of activities in the crystalline and in the dissolved state was 1.38. The effect of the medium on the reaction, as determined with cross-linked mixed crystals, was 1.36. Hence the activity of crystalline papain of both modifications towards BAEE was virtually 100%.

It has been ascertained under the dark-field microscope that there is no deterioration in crystals of either type during a run in the pH-stat and using both substrates.

DISCUSSION

The results show that papain crystals of two modifications are completely active towards two substrates. The activity of modification *A* towards AGEE seemed to be even slightly higher than that of the dissolved enzyme, but the authors do not feel justified in deciding whether or not this is some artifact.

As far as the authors are aware, this is the first case in which complete activity of enzyme crystals has been observed in a straightforward manner. This is partly due to the small size of the crystals and to the precautions taken to limit the diffusion barrier. As shown in the appendix, one can calculate $d_c = 8\,\mu$ for both substrates under the conditions used. Actually the *A* crystals were flat needles, about 1 μ thick. The *C* crystals were needles with a diamond-shaped cross-section. Most *C* crystals had a mean thickness of no more than 1 to 2 μ. Occasionally crystals had a mean thickness of 6 μ. In all cases the results were the same. Therefore the conditions prescribed by equation (1) were adhered to.

The authors did not obtain homogeneous crystal fractions of sufficient size to check Eqn. (1). In the literature one case is described in which the effect of crystal size has been examined: carboxypeptidase[3]. Calculation from the data mentioned in Table I of the appendix yields $d_c = 6\,\mu$, whereas the result actually found was 7.5 μ. This is a good agreement. The principles set forth in this paper can be applied to other cases. Using kinetic data of the substrates concerned from the literature (see APPENDIX) one can calculate for chymotrypsin $d_c = 5\,\mu$ and for ribonuclease 0.4 μ at pH 7 and 1.4 μ at pH 5. The smallest dimensions of the crystals actually used were 100 μ (ref. 2) and 50 μ (ref. 1), respectively. Therefore the crystals were too large.

* This data indicates that the mercuri-papain is accumulated neither at the surface of the crystals nor at the centre. The possibility that mercuri-papain and inactive papain are contained in separate crystals can be excluded in view of X-ray analytical results. Most papain preparations are mixtures of about equal amounts of active and inactive enzyme, differing only in thiol content. Crystals of mercuri-papain show a mercuri occupancy in the active site, as calculated from the difference Fourier map, which roughly corresponds with the thiol content. This indicates that mercuri-papain and inactive papain form mixed crystals.

It is possible to use Eqn. 8 of the appendix for an approximate correction of the activities observed for the crystal size. If this is done for the large crystals of carboxypeptidase with the calculated value of d_c in Eqn. 8, the activity of the three largest crystals is calculated to be 76%, 93% and 106% of the activity of the smallest crystals (which is only 0.3% of the activity of the dissolved enzyme). Applied to chymotrypsin this procedure indicates that the activity of crystalline chymotrypsin is 200% of that of the dissolved enzyme. In the case of crystalline ribonuclease-S, calculation indicates 90% activity at pH 7 and 140% at pH 5. This kind of correction is probably not reliable by more than a factor of two, owing to the uncertainty in the values of D_i and the shape of the crystal. Nevertheless these considerations show that chymotrypsin and ribonuclease-S could, like papain, be completely active in the crystalline state. A reinvestigation with smaller crystals, with poor substrates, *etc.* is desirable.

In future work, one should use Eqn. 1 (or 9) as a guide for finding the proper conditions under which diffusion is not rate limiting. One should avoid the necessity of using Eqn. 8 for the correction for the crystal size because on the one hand such a correction will not be a very accurate one, and on the other hand one cannot be certain in such a case whether the low activity is, in fact, entirely due to the large crystal size or to lower intrinsic activity inside the crystal.

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The nonlinearity shown in fig. 1 is abolished if inhibitory protein impurities are removed from papain samples previous to crystallization, by chromatography on a Sephadex G75 column.

APPENDIX

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In cases where crystals have the shape of flat plates with a thickness of d, the differential equation governing the internal concentration of substrate $[S]$ becomes (*cf.* ref. 1):

$$D_i \frac{d^2[S]}{dx^2} = \frac{k_{cat}[E_t][S]}{[S] + K_m} \qquad (2)$$

in which x is the distance from the middle of the crystal, $[E_t]$ is the total active enzyme concentration inside the crystal and the other parameters are those denoted in the main paper. This equation applies to steady-state conditions when the rate of diffusion (left-hand term) equals the rate of enzymic conversion (right-hand term). Since the free enzyme concentration $[E]$ equals:

$$[E] = \frac{K_m[E_t]}{[S] + K_m} \qquad (3)$$

Eqn. 2 can be converted into

$$\frac{d^2[S]}{dx^2} = \alpha^2[S] \qquad (4)$$

in which

$$\alpha^2 = \frac{k_{cat}[E]}{K_m D_i} \tag{5}$$

This equation can be solved when $[E]$ is independent of x, with the boundary conditions

$$[S] = [S_0] \text{ at } x = \pm \tfrac{1}{2}d \tag{6}$$

in which $[S_0]$ is the substrate concentration in the medium.

The solution of (4) and (6) is

$$[S] = [S_0]\frac{\cosh \alpha x}{\cosh \tfrac{1}{2}\alpha d}$$

from which the average substrate concentration $\langle S \rangle$ is calculated as

$$\langle S \rangle = \frac{2}{d}\int_0^{\frac{1}{2}d} [S]\mathrm{d}x = [S_0]\frac{\tanh \tfrac{1}{2}\alpha d}{\tfrac{1}{2}\alpha d}$$

Accordingly the enzymatic efficiency, e, of the crystals will be:

$$e = \frac{\langle S \rangle}{[S_0]} = \frac{\tanh \tfrac{1}{2}\alpha d}{\tfrac{1}{2}\alpha d} \tag{7}$$

In the limit of very thin crystal plates, $\alpha d \to 0$, $\tanh \frac{1}{2}\alpha d$ becomes equal to $\frac{1}{2}\alpha d$ and hence $e = 1$. For $\alpha d = 1$, e equals 0.92. This value of d is defined as the critical size, d_c. Hence

$$d_c = \frac{1}{\alpha} = \sqrt{\frac{K_m D_i}{k_{cat}[E]}} \tag{1}$$

and

$$e = \frac{\tanh \tfrac{1}{2}(d/d_c)}{\tfrac{1}{2}(d/d_c)} \tag{8}$$

These equations are valid only when $[E]$ is independent of x. This condition is adhered to when $[S] \ll K_m$, because then $[E] = [E_t]$. In such cases both Eqns. 1 and 8 are valid.

It is also adhered to when the crystals are no larger than the critical size, *i.e.*, when $[S] = [S_0]$ everywhere in the crystal. In that case $[E]$ is given by Eqn. 3. Insertion of Eqn. 3 into Eqn. 1 yields:

$$d_c = \sqrt{\frac{(K_m + [S_0])\ D_i}{k_{cat}\ \ [E_t]}} \tag{9}$$

Strictly speaking, insertion of this value of d_c into Eqn. 8 is not quite valid, because Eqn. 8 pertains to thick crystals in which $[E]$ is not constant if $[S] > K_m$. Nevertheless it can serve as a reasonable approximation for correcting activities for crystal size, at least up to $[S]/K_m = 70$ and $d/d_c = 40$, as a more rigorous computer calculation has shown.

It should be stressed that equation (9) is valid under steady-state conditions, even if the total enzyme concentration inside the crystal exceeds the substrate concen-

TABLE I

DATA USED FOR THE CALCULATION OF d_c VALUES

Enzyme	*Substrate*	$D \times 10^6$ ($cm^2 \cdot sec^{-1}$)	η_{rel} (*water* = 1)	*Substrate diameter* (Å)	$D_i \times 10^6$ ($cm^2 \cdot sec^{-1}$)	K_m (*mM*)	k_{cat} (sec^{-1})	$(E)_{total}$ (*mM*)	d_c (μ)
Carboxypeptidase	20 mM carbobenzoxyglycylphenylalanine	5	1.0	9	0.40	19*	0.9**	45	6
Papain	50 mM AGEE	6	2.0	7	0.31	450	1***	60	8.5
	20 mM BAEE	5	2.0	9	0.20	18	10	1.2	8
Chymotrypsin	2 mM acetyltyrosinehydrazide	6	2.4	8	0.25	22†	0.4	60	5
Ribonuclease	17 mM cytidine 2′,3′-phosphate, pH 7	5	2.7	9	0.15	11††	18	140	0.4
	17 mM cytidine 2′,3′-phosphate, pH 5	5	2.7	9	0.15	0.5††	1	140	1.4

* Value indicated by arrow at upper curve of Fig. 3 of ref. 1.

** Derived from the parameters of the dissolved enzyme (ref. 11) and the 300-fold lower apparent activity of the smallest crystals (Table V of ref. 3).

*** k_{cat}/K_m to be multiplied by 3.8, *i.e.*, the enhancing effect of 20% Na_2SO_4 upon the apparent activity.

† ref. 12.

†† ref. 13.

tration in the medium. Such conditions will cause the pre-steady-state period to be longer for the crystals than for the solution because the active sites (which act as sinks) will have to be filled with substrate molecules, *via* diffusion through the crystal, until quasi-equilibrium has been established. Once the steady state is established, the analytical concentration of substrate inside crystals satisfying Eqn. 9 will be higher than the substrate concentration outside, whereas the free substrate concentration inside will be equal to the concentration outside. The analytical substrate concentration will exceed the free concentration by an amount equal to the ES concentration in the crystal.

In the calculation of d_c with these equations, the uncertain factor is the value of D_i. If diffusion rates in water, D, of molecules of comparable size are utilised (*cf.* Table II of ref. 9), they have to be corrected for the following effects: (1) The viscosity of the suspension medium. For strong salt solutions this amounts to a correction factor of 2 to 3. Such a correction assumes that the molecules of the medium penetrate into the liquid of the crystals as far as the substrate molecules do. (2) The excluded volume, determined by the width of the slits of the liquid layers in the crystal and the effective diameter of the substrate molecules. A slit width of 15 Å is a reasonable estimate at the moment. This again amounts to a correction by a factor of 2 to 3 in several cases (*cf.* ref. 1). (3) The liquid layers in crystals are actually "solutions" of hydrophilic side chains. This is the most uncertain correction. In a number of protein crystals one can calculate a hydrophilic side-chain "molarity" of about 6 M. Somewhat arbitrarily this can be compared with a 6 M glycerol solution in which the high viscosity of the glycerol solutions ascribed to aggregation of the molecules due to hydrogen bonding replaces the effect of the attachment of the side chains to the protein backbone. This results in a correction by a factor of 5.

These corrections are admittedly crude. Fortunately they appear under the square root of Eqn. (1). Therefore values of d_c thus calculated may serve at least as a guide until more direct determinations of D_i become available (*cf.* ref. 10). The data used for the calculation of the various d_c values are collected in Table I.

REFERENCES

1 M. S. Doscher and F. M. Richards, *J. Biol. Chem.*, 238 (1963) 2399.
2 J. Kallos, *Biochim. Biophys. Acta*, 89 (1964) 364.
3 F. A. Quiocho and F. M. Richards, *Biochemistry*, 5 (1966) 4062.
4 H. Theorell, B. Chance and T. Yonetani, *J. Mol. Biol.*, 17 (1966) 513.
5 B. Chance, A. Ravilly and N. Rumen, *J. Mol. Biol.*, 17 (1966) 525.
6 B. Chance and A. Ravilly, *J. Mol. Biol.*, 21 (1966) 195.
7 J. Drenth, J. N. Jansonius, R. Koekoek, J. Marrink, J. Munnik and B. G. Wolthers, *J. Mol. Biol.*, 5 (1962) 398.
8 J. R. Kimmel and E. L. Smith, *Biochem. Prepn.*, 6 (1958) 61.
9 A. Polson, *Biochim. Biophys. Acta*, 140 (1967) 197.
10 H. W. Wyckoff, M. Doscher, D. Tsernoglou, T. Inagami, L. N. Johnson, K. D. Hardman, N. M. Allewell, D. M. Kelly and F. M. Richards, *J. Mol. Biol.*, 27 (1967) 563.
11 R. Lumry and E. L. Smith, *Discussions Faraday Soc.*, 20 (1955) 105.
12 W. E. M. Lands and C. Niemann, *J. Am. Chem. Soc.*, 81 (1959) 2204.
13 D. G. Herries, A. P. Mathias and B. R. Rabin, *Biochem. J.*, 85 (1962) 127.