KINETICS OF INHIBITION OF PAPAIN BY TLCK AND TPCK IN THE PRESENCE OF BAEE AS SUBSTRATE *

B. C. WOLTHERS

Laboratorium voor Structuurchemie, Bloemsingel 10, Groningen, The Netherlands

Received 21 November 1968

1. Introduction

The inhibition of papain by TPCK and TLCK was first reported by Shaw, Guia and Cohen [1]. Subsequently Husain and Lowe [2] demonstrated that TGCK reacted with papain to give an enzymically inactive product in which the sulphydryl group of cysteine-25 was substituted. Bender and Brubacher [3] were able to show a similar reaction of papain with TPCK by following the loss of the cysteine content of the enzyme after reaction with the inhibitor. The rate of inhibition increased with increasing pH, being dependent on a single prototropic group of pK 8.29 (25°), suggesting that a thiol anion was the reactive species. Similar results were obtained by Whitaker and Perez-Villaseñor [4] for the reaction with TLCK. This inhibitor reacts with cysteine-25 much faster than does TPCK, due to the fact that papain preferentially hydrolyses derivatives of arginine and lysine.

The effect of substrates on the inhibition rates of TPCK and TLCK is unknown. We therefore undertook a study to determine whether TPCK and TLCK have substrate-like properties. If these inhibitors behave like substrates, it would be expected that substrates should compete in the inhibition reaction.

The results suggest that TLCK has substrate-like properties and so could be used in X-ray diffraction studies of papain [5] as a model for an enzyme-substrate complex.

* Abbreviations used:
BAEE, benzoylarginine ethyl ester
EDTA, ethylenediaminetetraacetic acid
TGCK, tosylglycylchloromethylketone
TLCK, tosyllysylchloromethylketone
TPCK, tosylphenylalanylchloromethylketone

The rate of inactivation of papain by inhibitors in the presence of BAEE can be conveniently measured by the technique described by Sluyterman [6]. The rate of hydrolysis of BAEE is followed in a pH-stat. After addition of an inhibitor the concentration of active enzyme will diminish, thereby decreasing the observed rate. Traces are obtained from which the inhibition rate constants can be derived.

Sluyterman [6] found that with chloroacetic acid as inhibitor the inhibition rate constant remained the same at all BAEE concentrations. This suggests that: (1) free enzyme and enzyme-substrate complex have the same inhibition constants, and (2) that in the hydrolysis of BAEE by papain, which probably proceeds via an acylenzyme intermediate, acylation is the rate-limiting step. This last conclusion is supported by the results presented in this communication.

2. Experimental procedure

All reactions were carried out in a titration vessel at pH 7 and 25°. TPCK was added as an acetonitrile solution, resulting in an acetonitrile content of the reaction mixture of 3%.

All solutions contained 0.3 M KCl, to maintain a constant ionic strength, and 0.001 M EDTA. Cysteine was not used as an activator since it could interfere with the inhibition reaction. In the absence of cysteine, freshly prepared papain solutions show considerable activity which in the present experiments was found to decrease only slightly with time in the absence of inhibitors.

The procedure was as follows: to a BAEE solution of known concentration containing EDTA and KCl,

a certain amount of papain solution was added. After a few minutes of recorded run of the pH-stat, a known amount of inhibitor was added and the time noted on the record. The titration was then continued for at least 6 half-lives of active enzyme.

3. Evaluation of the titration curve

When e is the active enzyme concentration and i the inhibitor concentration, we can write for the inhibition reaction:

$$\frac{de}{di} = -k_i e i ,$$

where k_i is the inhibition rate constant.

Instead of e one can write v, the hydrolysis rate of the substrate, which is proportional to e according to the Michaelis-Menten equation. Moreover, when the inhibitor is present in large excess we can write:

$$\frac{dv}{dt} = -k_i' v ,$$

where $k_i' = k_i i$. Integrating we obtain:

$$v = v_0 \exp(-k_i' t) ,$$

where v_0 is the hydrolysis rate before addition of the inhibitor. Integrating again we obtain an expression for B_t, the quantity of base (equivalent to the quantity of substrate hydrolysed) added by time t after the addition of inhibitor:

$$B_t = \int_0^t v_0 \exp(-k_i' t) = \frac{v_0}{k_i'} [1 - \exp(-k_i' t)]$$

$$= B_\infty [1 - \exp(-k_i' t)] ,$$

where B_∞ ($= v_0/k_i'$) is the amount of base added at infinite time, when all the enzyme has become inactive. This last equation can also be written in the form:

$$\log B_\infty - \log(B_\infty - B_t) = \frac{k_i' t}{2.303} . \qquad (1)$$

Plots of the left-hand side of eq. (1) against time give straight lines from the slopes of which k_i' and hence k_i can be calculated.

The pH-stat trace should, after a certain time, remain horizontal but in practice it continues to rise at a slow, constant rate. This effect may be corrected for by assuming that the slow rise continues at the same rate throughout the experiment and extrapolating the final trace to zero time. This is shown in fig. 1 from which the method of obtaining B_∞ and B_t, necessary for the computation of k_i, can be seen.

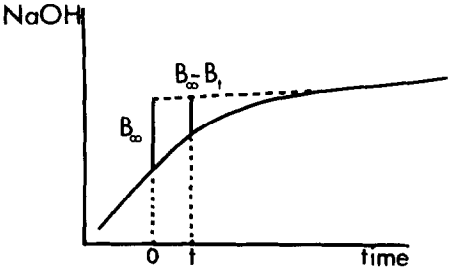

Fig. 1. Method of evaluation of titration curves.

4. Results and discussion

The values of the inhibition constants, k_i, for TLCK and TPCK obtained in this work are shown in table 1. When these values of k_i are plotted against $K_m/(K_m + [S])$, equal to $[E]/([E] + [ES])$, as suggested by Sluyterman [6], straight lines are obtained which may be extrapolated to $[S] = \infty$ ($K_m/(K_m + [S]) = 0$) to give k_i for the complex ES, and to $[S] = 0$

Table 1
Inhibition rate constants for TLCK and TPCK at different BAEE concentrations.

TLCK		TPCK	
BAEE (M)	k_i (M^{-1} sec^{-1})	BAEE (M)	k_i (M^{-1} sec^{-1})
0.0078	910	0.0097	57
0.015	660	0.0194	67
0.031	450	0.0388	59
0.033	450	0.0776	65
0.063	300		
0.066	280		
0.13	160		

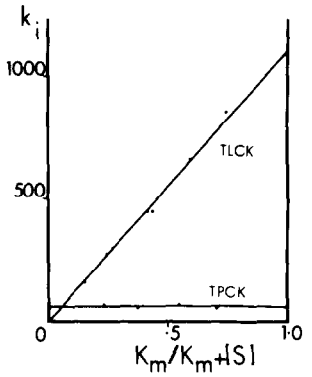

Fig. 2. Inhibition rate constants, k_i, for TLCK and TPCK at different BAEE concentrations. Extrapolations to BAEE concentrations = 0 and ∞ ($K_m/(K_m + [S]) = 1$ and 0).

$(K_m/(K_m + [S]) = 1)$ to give k_i for the free enzyme. This is shown in fig. 2. In these calculations K_m was taken as 0.0225 M, at pH 7, from our own measurements; this value is in agreement with the literature [7].

The results obtained here are very surprising both for TPCK and for TLCK. The inhibition rate constants for TPCK are independent of the BAEE concentration, as are those for chloroacetic acid, thus confirming that the acylation step for BAEE is rate-limiting. Moreover, the inhibition reaction is not hindered by the presence of BAEE at the active centre. One can therefore conclude: (1) that TPCK cannot be regarded as a substrate-like inhibitor and (2) that there must be a lot of room around cysteine-25 at the active site, since the complexing of BAEE to papain does not hinder the approach of the bulky TPCK molecule. That this is indeed so is confirmed by the model of the papain active site, constructed from the results of X-ray analysis [5].

Finally it is evident that the tosyl group does not contribute much to the binding or reactivity of TPCK. Tosylarginine methyl ester is, however, a good substrate while arginine methyl ester is not, showing that the tosyl group is only important in the later stages of the catalytic process. A similar result was obtained with chymotrypsin [8].

As has been pointed out by Whitaker and Perez-Villaseñor [4], the TPCK reaction is possible because papain is unable to exclude this reagent from its active site.

The results with TLCK are quite different. The lower the substrate concentration the higher is the inhibition rate constant. Therefore TLCK probably occupies the same site as the normal substrate and so may be said to possess substrate-like properties. This is supported by the observation that TLCK reacts with free enzyme at approximately 20 times the rate at which TPCK reacts (k_i for TPCK ≈ 60 and for TLCK $\approx 1120\ M^{-1}\ sec^{-1}$).

Extrapolation to $[S] = \infty$ gives the k_i value for the ES complex which is $k_i \approx 0$. This result is subject to the 10% error in the measurement of k_i values but nevertheless establishes that k_i for the ES complex for TLCK is smaller than that for TPCK. This suggests that TLCK reacts in a substrate-like fashion whereas the TPCK reaction does not resemble the normal catalytic process.

Acknowledgements

I am grateful to Dr. J.Drenth for suggesting the use of TPCK and TLCK for X-ray diffraction studies, I also wish to thank Dr. L.A.Ae.Sluyterman for suggesting the method of evaluating the titration curve and Dr. J.N.Jansonius for many useful suggestions. The present investigation was carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

References

[1] E.Shaw, M.M.Guia and W.Cohen, Biochemistry 4 (1965) 2219.
[2] S.S.Husain and G.Lowe, Chem. Commun. (1965) 345.
[3] M.L.Bender and L.J.Brubacher, J. Am. Chem. Soc. 88 (1966) 5880.
[4] J.R.Whitaker and J.Perez-Villaseñor, Arch. Biochem. Biophys. 124 (1968) 70.
[5] J.Drenth, J.N.Jansonius, R.Koekoek, H.M.Swen and B.G. Wolthers, Nature 218 (1968) 929.
[6] L.A.Ae.Sluyterman, Biochim. Biophys. Acta 151 (1968) 178.
[7] E.L.Smith and M.J.Parker, J. Biol. Chem. 233 (1958) 1387.
[8] D.W.Ingles and J.R Knowles, Biochem. J. 108 (1968) 561.