Biochimica et Biophysica Acta, 309 (1973) 175–180

BBA 66886

AN ACTIVE-SITE TITRATION METHOD FOR IMMOBILIZED TRYPSIN

JAMES R. FORD, ROBERT P. CHAMBERS AND WILLIAM COHEN
Departments of Biochemistry and Chemical Engineering, Tulane University, New Orleans, La. 70112 (U.S.A.)

(Received December 4th, 1972)

SUMMARY

An active-site titration method for immobilized trypsin with *p*-nitrophenyl *p'*-guanidinobenzoate (NPGB) employing a recirculation reactor system is described. Trypsin, covalently linked to 35 and 150 μm diameter porous glass particles was titrated by this procedure, analyzed for protein content, and then compared with soluble trypsin. Analysis of the titration results indicates that the amount of *p*-nitrophenol produced by the burst is equal to the amount of active immobilized trypsin. This active site titration determines the absolute amount of active immobilized enzyme and is preferable to either total protein analysis or kinetic assays for characterization of immobilized enzymes.

INTRODUCTION

Many enzymes have been covalently linked to insoluble matrices with retention of enzymic activity[1]. However, routine methods for the reliable determination of the amount of active enzyme immobilized on the matrix are not currently available. The goal of this paper is the development of a readily generalizable active-site titration method which will accurately determine the amount of active trypsin covalently bound to porous glass.

The basis for active site titration methods lies in the specific stoichiometric reaction of enzymes with active-site-directed reagents[2–6]. An excellent active site titrant for trypsin has been found to be *p*-nitrophenyl *p'*-guanidino-benzoate (NPGB)[7]. The active site titrant NPGB is a substrate-like reagent which rapidly and stoichiometrically reacts with the active site of trypsin in the same manner as a specific substrate. However, the reaction yields a relatively stable acylated enzyme with the concurrent production of a "burst" of *p*-nitrophenol. The amount of spectrophotometrically determined *p*-nitrophenol in the burst is equal to the amount of active enzyme.

In this report we describe a reliable method for titration of glass-immobilized

Abbreviation: NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate.

trypsin with NPGB by application of the recirculation reactor assay system developed in this laboratory[8] and compare its use to other methods for determining active immobilized enzyme.

MATERIALS AND METHODS

Immobilized trypsin

Trypsin (bovine pancreas, Worthington, TRL) was immobilized on 2000 Å pore diameter 96% silica glass (Corning CPG-10-2000) by a modification of the aryl-azo technique of Weetall[9].

Protein analysis

The amount of trypsin protein immobilized on the porous glass was determined by quantitative amino acid analysis according to Spackman *et al.*[10]. Samples (approx. 50 mg) of immobilized trypsin were thoroughly washed with deionized water and 0.1 M phosphate buffer (pH 7.5), dried at 90 °C, weighed and hydrolyzed for 48 h in 6 M HCl at 110 °C. Soluble trypsin (1 mg) was similarly treated. The protein content of these hydrolysates was calculated from the aspartic acid content. Trypsin was assumed to have 22 residues of aspartic acid per mole[11].

RESULTS

Active site titration

The titration is carried out in the recirculation system, Fig. 1, with the volume adjusted to about 5–6 ml by employing a small (approx. 1 ml) reservoir. The reactor, removed from the system, is empty except for a sample of approx. 50 mg of thoroughly-washed glass-immobilized enzyme. The remainder of the system is filled with 0.03 mM NPGB solution, freshly prepared by diluting 3 mM NPGB in dimethylsulfoxide with 50 mM veronal buffer, pH 8.3, and the contents are recirculated at 10 ml/min. The Cary 16K spectrophotometer is zeroed at 410 nm with a range of 0.1 *A* and the slow rate of pre-burst hydrolysis is recorded.

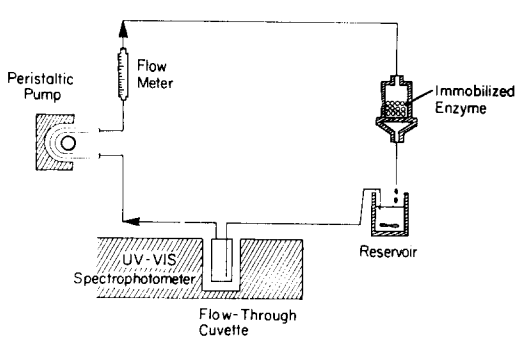

Fig. 1. Recirculation titration system.

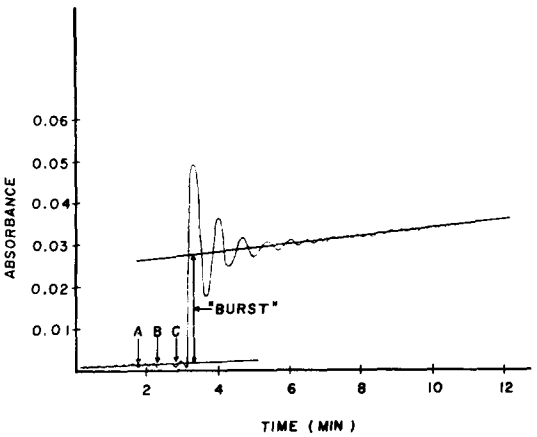

Fig. 2. Active-site titration recorder trace. 62.1 mg of 35 μm particle diameter glass-bound trypsin titrated with 5.1 ml of 0.03 mM NPGB in 50 mM veronal buffer, pH 8.3. A, pump off; B, reactor plugged into system; C, pump restarted; Burst, 0.0265 A.

The active site titration is initiated by stopping the pump, inserting the reactor containing the enzyme into the system, starting the pump up slowly, inverting the reactor for a short while to purge it of air and finally increasing the recirculation pump speed to a flow rate of 10 ml/min. The increased absorption due to the burst and the following slow post-burst hydrolysis are traced on the strip recorder. Fig. 2 shows a recorder trace from the titration of 62.1 mg of 35 μm particle diameter glass-bound trypsin. The solution contains a large excess of NPGB over its stoichiometric requirement; thus, the initial oscillatory behavior observed is due to the production of a small "plug" of *p*-nitrophenol as a result of the rapid reaction of an approximately stoichiometric volume of titrant solution with the enzyme as it passes thru the reactor. The oscillations are quickly damped out as this plug disperses and the *p*-nitrophenol concentration throughout the recirculation system becomes equal. The magnitude of the burst is obtained by simple extrapolation of pre- and post-burst hydrolysis rates to the point of the initial enzyme–titrant contact.

The fluid is pumped out of the system and its volume is accurately measured. The immobilized enzyme is carefully removed from the reactor, dried at 90 °C and weighed. The moles of active trypsin may be calculated from the following equation:

$$\mu\text{moles enzyme/g glass} = 10^6 \, \Delta A \, v/\varepsilon_{410\text{ nm}} \, w \, b \qquad (1)$$

where ΔA = burst absorbance; v = total fluid volume (ml); $\varepsilon_{410\text{ nm}}$ = extinction coefficient of *p*-nitrophenol ($1.66 \cdot 10^4$ M^{-1} cm^{-1}); w = weight of glass (mg); and b = cuvette path length (cm). From Fig. 2 the burst is 0.0265 A, v is 5.1 ml and a value of 0.13 μmole active trypsin/g glass was calculated.

Increasing the concentration of NPGB five times did not affect the size of the burst. Elimination of dimethylsulfoxide also did not affect the results. Active site titrations of soluble trypsin were performed according to Chase and Shaw[7].

Comparison of results

Trypsin, covalently linked to 35 and 150 μm porous glass particles, was titrated

TABLE I

ANALYSIS OF POROUS GLASS-IMMOBILIZED TRYPSIN

Trypsin	*μmoles trypsin/g glass*		*μmole active trypsin/ μmole total protein*	*Fractional activity retained upon binding*
	*Apparent** *A*	*True*** *B*	*C*	*D*
Soluble	—	—	0.70	—
35-μm immobilized	0.48	0.13	0.27	0.39
150-μm immobilized	0.63	0.14	0.23	0.33

* Calculated from amino acid analysis data.
** Calculated from active-site titration data.

with NPGB, analyzed for protein content, and then compared with soluble trypsin.

Table I gives data on the amount of trypsin bound to glass. Apparent values calculated from amino acid analyses are shown in Column A and true values calculated from active site titrations are shown in Column B. Columns C and D show the fraction of total protein that is active and the fraction of initially active soluble protein that retained activity upon binding, respectively. One may readily see that about two-thirds of the initial enzyme activity was lost in the process of binding to the glass.

DISCUSSION

Chase and Shaw[7] added NPGB to a solution of soluble trypsin resulting in an immediate burst of *p*-nitrophenol followed by slow post-burst hydrolysis of the reagent. This post-burst hydrolysis was found to be primarily nonspecific esterolysis catalyzed by other parts of the protein, rather than either deacylation and subsequent turnover of the acyl enzyme, or spontaneous hydrolysis. However, the implementation of active site titration methods for immobilized enzymes results in both pre-burst and post-burst hydrolysis. The pre-burst hydrolysis occurs in the absence of immobilized enzyme and thus represents spontaneous hydrolysis. The rate of post-burst hydrolysis is significantly greater than the pre-burst rate and consists of the sum total of spontaneous hydrolysis, turnover of the acylated enzyme, nonspecific protein-catalyzed esterolysis, and glass-catalyzed esterolysis. The continuously monitored recirculation reactor system permits accurate extrapolation of these rates to the time of the burst and also provides the sensitivity to give accurate results at active enzyme concentrations of $1 \cdot 10^{-6}$ moles trypsin/l fluid volume, which is similar to the minimum accurately determinable active enzyme concentration quoted by Chase and Shaw[7] for soluble trypsin. This degree of accuracy, precision and ease could not have been accomplished by simply stirring the glass-bound enzyme in a NPGB solution, removing the immobilized enzyme by centrifugation and filtration, and measuring the absorbance change of the NPGB solution, since it is not possible to accurately estimate the post-burst hydrolysis contribution to the total absorbance during the time required by centrifugation, *etc.*

The reaction of the active site titrant, *S*, with trypsin proceeds as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+\ P_1}{ES'} \xrightarrow{k_3} \underset{+\ P_2}{E} \qquad (2)$$

When $[S]_0 \gg [E]_0$, Bender *et al.*[12] have shown that the concentration of *p*-nitrophenol in the burst, π, is related to the active enzyme concentration, $[E]_0$, by the following equation:

$$\pi = \frac{[E]_0 [k_2/(k_2 + k_3)]^2}{(1 + K_m/[S]_0)^2} \tag{3}$$

Chase and Shaw[7] showed that for NPGB–soluble trypsin titrations $k_2 \gg k_3$ and $[S]_0 \gg K_m$, thus π is equal to $[E]_0$. The absence of an effect of NPGB on the titration of immobilized trypsin demonstrates that π is independent of $[S]_0$; thus $[S]_0 \gg K_m$. The apparent k_2 is much larger than k_3, when k_3 is taken as the maximum possible contribution that turnover could have in the post-burst hydrolysis. We therefore reach the same conclusion that was obtained for the soluble enzyme: the amount of *p*-nitrophenol in the burst, π, is equal to the amount of active trypsin immobilized in the glass.

Active-site titration techniques have many advantages over either total protein or kinetic assay methods for the determination of active immobilized enzyme. As seen from Table I, 61–67% of the initially active trypsin has lost its activity upon binding; thus total protein is neither an absolute measure of active enzyme nor an accurate relative measure of active enzyme.

The use of kinetic assay methods requires several assumptions regarding the kinetic parameters of the immobilized enzyme and the influence of diffusion on the results. For many substrate–immobilized enzyme combinations the rapid rate of reaction provides sizable concentration gradients both outside the immobilizing matrix and within its pores. These concentration gradients greatly retard the rate of reaction, even when the substrate concentration within the pores is much greater than the immobilized enzyme K_m (ref. 13). This diffusional effect serves to decrease the apparent active enzyme concentration.

In order to obtain valid active enzyme concentrations from a kinetic assay, the k_{cat} for the immobilized enzyme must be known. However, the effects of the micro-environment and the immobilization process itself cause the kinetic parameters of the immobilized enzyme to frequently differ from those of the soluble enzyme. The shift in k_{cat} of the immobilized enzyme as a result of changes in the micro-environmental pH (pH optima shift) has been well documented[14].

Therefore, a routine active-site titration technique is the preferred method for the determination of the activity of immobilized enzymes. It is believed that this titration method for trypsin is quite general and can be readily extended to other immobilized enzymes.

ACKNOWLEDGMENTS

The authors wish to thank the Advanced Technology Applications Division of the RANN program of NSF (Grant GI-34974) and The Bush Foundation for support of this research.

REFERENCES

1 Goldman, R., Goldstein, L. and Katchalski, E. (1971) in *Biochemical Aspects of Reactions on Solid Supports* (Stark, G. R., ed.), pp. 1–78, Academic Press, New York
2 Balls, A. K. and Jansen, E. F. (1952) *Adv. Enzymol.* 13, 321–343

3 Schoellman, G. and Shaw, E. (1962) *Biochem. Biophys. Res. Commun.* 7, 36–40
4 Shaw, E., Mares-Guia, M. and Cohen, W. (1965) *Biochemistry* 4, 2219–2224
5 Baker, B. R. (1967) *Design of Active-Site-Directed Irreversible Enzyme Inhibitors*, pp. 122–153, John Wiley and Sons, New York
6 Mares-Guia, M., Shaw, E. and Cohen, W. (1967) *J. Biol. Chem.* 242, 5777–5781
7 Chase Jr, T. and Shaw, E. (1967) *Biochem. Biophys. Res. Commun.* 29, 508–514
8 Ford, J. R., Lambert, A. H., Cohen, W. and Chambers, R. P. (1972) *Biotechnol. and Bioeng. Symp.* No. 3, 267–284
9 Weetall, H. H. (1969) *Science* 166, 615–617
10 Spackman, D., Stein, W. and Moore, S. (1958) *Anal. Chem.* 30, 1190–1206
11 Dayhoff, M. O. (1969) *Atlas of Protein Sequence and Structure*, pp. D115–D116, National Biomed. Res. Found., Md.
12 Bender, M. L. et al. (1966) *J. Am. Chem. Soc.* 88, 5890–5913
13 Lambert, A. H., Ford, J. R., Cohen, W. and Chambers, R. P. (1971) *The Influence of Diffusion on the Kinetic Behavior of Enzymes Immobilized in Porous Solids*, presented at San Francisco AIChE Meeting, Dec. 1971
14 Katchalski, E., Silman, I. and Goldman, R. (1971) *Adv. Enzymol.* 34, 445–536