A "REVERSE BURST" ACTIVE SITE TITRATION PROCEDURE FOR HUMAN CARBONIC ANHYDRASE B

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Summary: Between pH 7 and pH 10.5, p-nitrophenyl p-sulfamyl benzoate (PNP-SAB) binds very strongly to human carbonic anhydrase B (dissociation constant on the order of 10^{-9} M or less at pH 7.5), but is not hydrolyzed by the enzyme. Because the binding is essentially stoichiometric under readily accessible conditions, this ester may be used as an active site titrant, by measuring the rapid hydrolysis of excess unbound PNP-SAB catalyzed by an added nucleophile ("reverse burst").

The development of rapid and convenient procedures for the titration of enzyme active sites remains a major objective of modern enzymology.[1] Titration procedures involving the measurement of absorbance "bursts" due to the release of chromogenic products from the stoichiometric reactions of suitable substrates with enzyme active sites have been described in a number of cases.[2] In the present article we wish to report the use of the ester p-nitrophenyl p-sulfamyl benzoate in a complementary approach in the determination of the active site normality of human carbonic anhydrase B (HCAB) solutions, a method which we term "reverse burst" titration.

Materials and Methods

Carbonic anhydrase was prepared from human erythrocytes by affinity

chromatography.[3] The B isozyme which was purified by the ion exchange method of Armstrong et al.[4] was electrophoretically homogeneous, exhibited the correct amino acid composition, and catalyzed the hydrolysis of 2-hydroxy 5-nitro-α-toluenesulfonic acid sultone[5] with a second order rate constant of 7.6×10^5 $M^{-1}sec^{-1}$ in 0.1 M Tris-sulfate buffer, pH 7.7, 25°.

The sultone substrate (Eastman) was recrystallized from ethanol and all organic solvents were dry-distilled prior to use. p-Nitrophenyl p-sulfamylbenzoate was prepared by DCC coupling of p-nitrophenol with p-sulfamyl benzoic acid in DMF. After precipitating the ester with excess H_2O, it was recrystallized from ethanol. The pure ester melted at 169-170° (uncorr.). Anal. Calcd for $C_{13}H_{10}N_2O_6S$: C 48.46; H, 3.1; N, 8.69; S, 9.95. Found: C, 48.57; H, 3.09; H, 8.74; S, 9.90.

The concentration of p-nitrophenol was calculated using $\epsilon_{400} = 18,100$ for the phenolate species and a pK for p-nitrophenol of 7.15.[6] The concentration of enzyme solutions was also measured spectrophotometrically, $\epsilon_{280} = 48,900$.[7]

Results and Discussion

We have discovered that p-nitrophenyl p-sulfamyl benzoate, PNP-SAB, is bound tightly in a stoichiometric fashion to HCAB but is hydrolyzed slowly, if at all, by the enzyme over the pH range 7-10.5. For example, in Tris-sulfate buffer, pH 7.4, the addition of 1×10^{-6} M HCAB to 3.5×10^{-5} M PNP-SAB resulted in no increase in the overall rate of p-nitrophenol release. The addition of increased amounts of enzyme was without effect until approximately equimolar amounts of enzyme and ester were present, at which point the buffer-catalyzed hydrolysis of the ester ceased, evidently due to the formation of an hydrolytically stable complex between PNP-SAB

and the enzyme. Over the pH range 7-10.5 by adding nucleophiles which catalyze the hydrolysis of PNP-SAB to solutions containing HCAB and excess PNP-SAB, it is possible to obtain a "burst" due to the rapid release of p-nitrophenolate from that part of the substrate which is not bound to the enzyme's active site ("reverse burst"). By simple subtraction of the concentration of the unbound substrate determined in this manner from the known initial (total) concentration of the substrate, the active site normality of the HCAB solution can be established.

In all of the cases examined it was found that an increase in the concentration of added nucleophile sufficient to cause a conveniently rapid hydrolysis of uncomplexed ester was accompanied by a significant, though much slower, hydrolysis of the initially complexed ester. Thus, in order to determine the concentration of ester unbound at time (t) = 0, it was necessary to extrapolate back to t = 0 from a point early in the hydrolysis of the initially complexed ester when p-nitrophenol release was very nearly zero-order, as is illustrated in Figure 1.*

With the nucleophiles tested the hydrolysis of unbound ester proceeded according to apparent first-order kinetics and occurred at the same rate as in the absence of enzyme. Among these nucleophiles 10 mM hydroxylamine in 0.1 M piperazine-N,N'-bis(2-ethanesulfonic acid) (Pipes), pH 7.5, proved to give the most accurate and reproducible results as illustrated in Table 1. Under these conditions, at 25.0° the hydrolysis of uncomplexed PNP-SAB was complete in about 7 min, and the results are accurate and

*Whether the slow hydrolysis of the initially complexed ester involves reaction on the enzyme or corresponds to the slow dissociation of the enzyme·PNP-SAB complex, followed by rapid scavenging by the added nucleophile is being investigated in our laboratory.

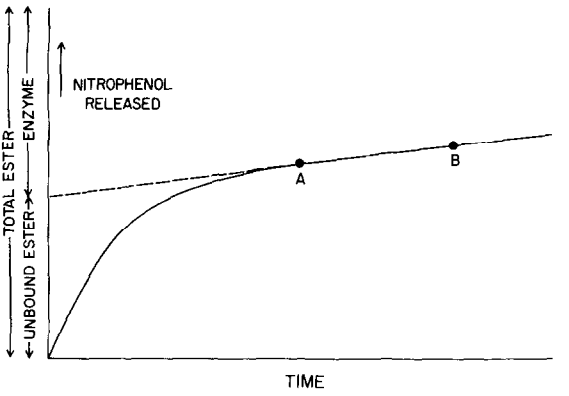

Figure 1. --Schematic representation of a "reverse burst" active site titration of HCAB with PNP-SAB. At point A, the hydrolysis of unbound ester is complete. Between points A and B the much slower hydrolysis of bound ester appears to approximate zero-order kinetics, enabling the concentration of unbound ester to be determined at time = 0. Thus, [Enzyme active sites] = [Total Ester] [Unbound Ester).

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reproducible to within 1% at an enzyme concentration of 10 μM.

An attempt to measure the dissociation constant, K_d, for the complex of PNP-SAB with HCAB at pH 7.5 by determining the extent of the concomitant inhibition of the enzyme-catalyzed hydrolysis of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone failed because PNP-SAB bound almost stoichiometrically, even at very low enzyme concentrations. The value of K_d appears to be on the order of 10^{-9} M or less.** Because K_d is very small it is possible not only to carry "reverse burst" active site titrations with PNP-SAB but also to employ this compound as an inhibitor in rate assay measure-

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**The inhibition of carbonic anhydrase action by several alkyl esters of p-sulfamyl benzoate has been measured and K_d values for the enzyme-inhibitor complexes as low as about 10^{-10} M have been measured.[8]

TABLE 1

"REVERSE BURST" TITRATION OF HCAB IN VARIOUS BUFFER SYSTEMS AT 25.0°

Buffer[b]	pH	Added Nucleophile	Enzyme Conc. (M)[a]	Active Site Conc. from Titration (M)
Pipes (0.1 M)	7.5	NH_2OH (10 mM)	5.4×10^{-6}	5.3×10^{-6} 5.4×10^{-6}
Pipes (0.1 M)	7.5	NH_2OH (10 mM)	1.07×10^{-5}	1.08×10^{-5} 1.08×10^{-5}
Imidazole (0.125 M, free base)	7.7	-	1.10×10^{-5}	0.95×10^{-5} 1.20×10^{-5}
Borate (0.020 M)	10.3	-	1.56×10^{-5}	1.54×10^{-5} 1.60×10^{-5}

[a] Measured spectrophotometrically using $\epsilon_{280} = 48,900$.

[b] The final CH_3CN concentration in the reaction solutions was 0.8 (v/v) percent.

ments which can lead to the determination of the enzyme active site concentration, as illustrated in Figure 2 where 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone is the assay substrate.* However, the "reverse burst" technique is far more convenient.

In preliminary studies we have found that the accurate active site titration of HCAB can be achieved by the "reverse burst" method using PNP-SAB even in the presence of a comparable concentration of another

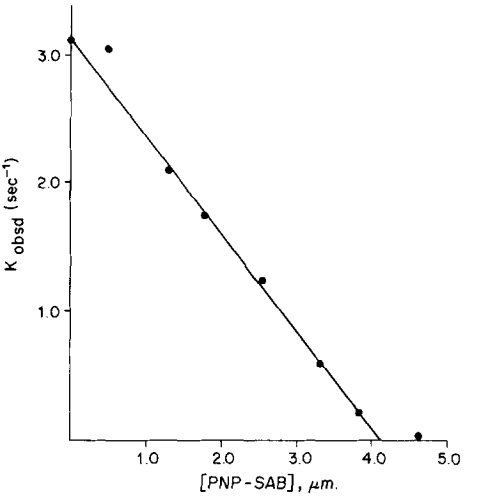

Figure 2.--Active site titration of HCAB with PNP-SAB employing a rate assay with 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone. The hydrolysis of the latter substrate was followed at 410 nm on an Aminco-Morrow stopped-flow spectrophotometer. The buffer was 0.1 M Tris-sulfate, pH 7.68, 1% CH_3CN, 25.0°. [Enzyme] = 4.11 x 10^{-6} M.

*The use of an inhibitor in conjunction with a rate assay has been conventionally applied in measurements of the active site normalities of carbonic anhydrase solutions, as exemplified by Kernohan's titration of bovine carbonic anhydrase solutions with ethoxazolamide.[9]

enzyme, α-chymotrypsin, which reacts with this ester. We are actively continuing our investigation of the interaction of PNP-SAB and its structural isomers with other carbonic anhydrase species.

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