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Comparison of Resorufin Acetate and *p*-Nitrophenyl Acetate as Substrates for Chymotrypsin

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Resorufin acetate is shown to be an attractive substrate to use with chymotrypsin since the absorbance of the product is several times more intense than that formed by the widely used *p*-nitrophenyl acetate. Furthermore, under the right conditions, resorufin acetate allows convenient observation of the burst reaction by conventional spectrophotometry. The steady-state k_{cat} values for chymotrypsin-catalyzed hydrolysis of resorufin acetate and *p*-nitrophenyl acetate are virtually the same, as expected for a rate-limiting deacylation step involving an identical intermediate from both substrates. Stopped-flow studies show that the maximal bursts of product from both substrates are again (in molar terms) about the same. When chymotrypsin is presented with a mixture of both substrates, the monitoring of reaction with resorufin acetate (at 571 nm) is not interfered with by simultaneous hydrolysis of *p*-nitrophenyl acetate. Under these conditions, *p*-nitrophenyl acetate is shown to increase the burst rate constant for acylation of the enzyme by resorufin acetate, demonstrating unequivocally that *p*-nitrophenyl acetate can bind to chymotrypsin elsewhere than in the active site.

INTRODUCTION

In previous work, enzymes with an esterase ability have been studied using various chromogenic substrates and substrate analogs. For example, we have extensively studied the kinetics of the reaction between cytosolic aldehyde dehydrogenase and certain *p*-nitrophenyl esters, carbonates, and lactones (*1–3*), and we have used *p*-nitrophenyl dimethylcarbamate to provide a label for identifying the enzyme's catalytically essential residue (*4*). We have developed a very useful cyclic carbamate that provides a covalently linked *p*-nitrophenol "reporter group" and used it to probe the nature of the active site in chymotrypsin (*5*) and aldehyde dehydrogenase (*6*). Recently we have observed that resorufin acetate (7-acetoxy-3*H*-phenoxazin-3-one) [which has been used in the fluorometric determination of cellulase activity (*7*)] is an excellent substrate for aldehyde dehydrogenase and that the reaction is dramatically accelerated by the presence of the enzyme's cofactors (*8*). The structures of resorufin acetate and of its chromophoric hydrolysis product (shown as a symmetrical resonance hybrid) are given in Scheme 1.

The purpose of the present work was to examine resorufin acetate as a substrate for chymotrypsin using stopped-flow and conventional spectrophotometry. The reaction of the enzyme with *p*-nitrophenyl acetate is of course well known and furnished one of the classic early demonstrations of "burst" kinetics and of the existence of a covalent acyl-enzyme intermediate (*9*). It was anticipated that similar

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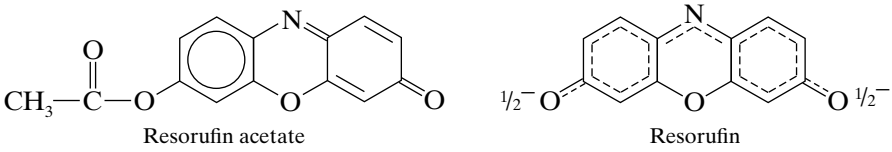

Resorufin acetate Resorufin

SCHEME 1

experiments with resorufin acetate would allow interesting observations and conclusions to be made, and these are reported here.

MATERIALS AND METHODS

Resorufin acetate. This compound was prepared from the sodium salt of resorufin (Aldrich Chemical Co., Inc.) as described by Nietzki *et al.* (*10*) and recrystallized from ethanol. δ ($CDCl_3$): 2.36 (s, 3H), 6.33 (s, 1H), 6.84–6.89 (d, 1H), 7.11–7.15 (d, 1H), 7.15 (s, 1H, superimposed), 7.41–7.45 (d, 1H), 7.78–7.81 (d, 1H). $C_{14}H_9NO_4$ confirmed by mass spectrometry (m/z = 255.052719).

Conventional spectrophotometry. A Varian Cary 1 spectrophotometer was used to monitor the hydrolysis of resorufin acetate (at 571 nm) and *p*-nitrophenyl acetate (at 399 nm) at 25°C in 50 mM Tris/HCl buffer, pH 8.5. α-Chymotrypsin (Aldrich, type II, from bovine pancreas) was added as 0.1 ml of a 10 mg/ml solution in the same buffer, and substrate was added as 0.1 ml of an acetone solution. The final enzyme concentration was 13.3 μM and the final volume was 3 ml. In all cases, the measured steady-state rates were corrected for the rate of spontaneous hydrolysis of substrate. Each assay was performed in duplicate or triplicate with very satisfactory agreement.

Stopped-flow spectrophotometry. This was carried out using a Hi-Tech Scientific instrument at 25°C. One syringe contained chymotrypsin in 50 mM Tris/HCl buffer, pH 8.6; the other syringe contained a mixture of 4.5 ml of this buffer and 0.5 ml of substrate in acetone. Substrate concentrations referred to below are those after mixing; the enzyme concentration (again after mixing) was 13.3 μM. Stopped-flow traces were computer-fitted to an exponential burst followed by a straight line, using software supplied by Hi-Tech Scientific. All runs were repeated at least five times with very close agreement. Typically the standard deviation in the calculated steady-state rate was less than 2.5%, and the error in the burst size and rate constant was even smaller.

RESULTS AND DISCUSSION

Figure 1A shows the uv/visible spectrum of resorufin acetate (25 μM). After complete hydrolysis at pH 7.4 [catalyzed by aldehyde dehydrogenase, which is an effective esterase (*1*)] the resulting resorufin solution has a strikingly beautiful

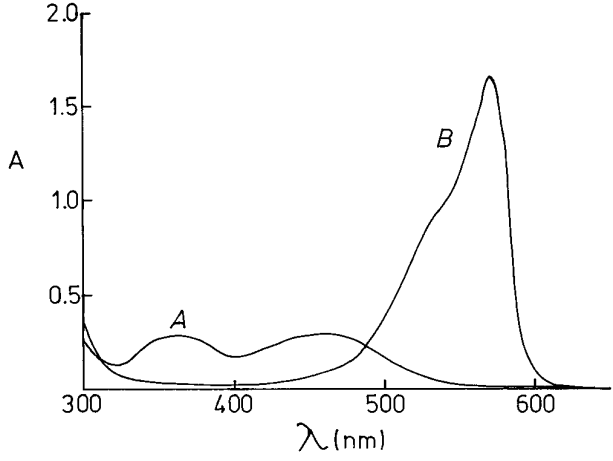

FIG. 1. Absorbance spectra of (A) resorufin acetate (25 μM) and (B) resorufin (25 μM) in 50 mM sodium phosphate buffer, pH 7.4.

intense pink color and gives spectrum B. The single large unsymmetrical peak has a λ_{max} of 571 nm and its size is pH dependent, with a pK_a of approximately 5.8 and a maximum extinction coefficient at higher pH of 69,700. [Note that standard resorufin solutions are best prepared as described above by hydrolysis of recrystallized resorufin acetate, since commercial resorufin (sodium salt) is impure and contains approximately 25% "inert inorganic material" (*11*).] The corresponding data for *p*-nitrophenol are λ_{max} 399 nm, pK_a 7.1 (*5*), and ε 18,320 (*12*). At pH 8.5–8.6 (used in this work) the ratio of extinction coefficients is 4.2, showing that resorufin acetate is potentially a considerably more sensitive substrate than the widely used *p*-nitrophenyl acetate.

Chymotrypsin was indeed found to catalyze the hydrolysis of resorufin acetate; a typical progress curve of an experiment using a conventional spectrophotometer is shown in Fig. 2. Under these conditions ([*E*] = 13.3 μM; [S] = 75 μM, pH 8.5), a fairly slow burst of resorufin liberation can clearly be seen, followed by a linear steady-state rate (which was approximately 12 times as great as the rate of spontaneous hydrolysis in the absence of enzyme). In the figure, the arrow shows the point at which the substrate was added as rapidly as possible; the trace then shows a small "blip" as the cell compartment was closed, followed smoothly by the burst. By back-projecting the linear part of the trace and subtracting the observed absorbance, a value for the first-order rate constant can easily be calculated; for this experiment it was 0.13 s^{-1}, but later the rate constant was found to be strongly dependent on substrate concentration. In undergraduate teaching laboratories without access to a stopped-flow instrument, the system used here would conveniently provide an excellent demonstration of the occurrence of a burst in chymotrypsin-catalyzed reactions.

The fact that resorufin acetate is a substrate for chymotrypsin implies that the resorufin moiety occupies the hydrophobic binding pocket [the S_1 site (*13*, pp.

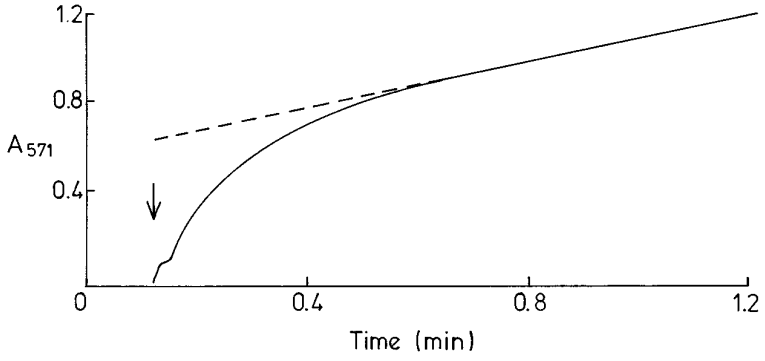

FIG. 2. Progress curve for the chymotrypsin-catalyzed hydrolysis of resorufin acetate (75 μM) at pH 8.5, obtained using a conventional spectrophotometer. The arrow shows the point at which substrate was added as rapidly as possible to a mixture of enzyme and buffer.

29–30)], which interacts with the side chain of amino acids such as Phe, Tyr, or Trp when the enzyme acts on its natural substrates. The dimensions of this pocket have been estimated as 10–12 × 5.5–6.5 × 3.5–4.0 Å (*14*). Resorufin acetate is essentially a planar molecule and its length and breadth can be calculated as 12.6 and 5.1 Å, respectively (*15*). The distance from the carbonyl group where enzymatic attack occurs to the far end of the molecule is approximately 10.5 Å. Thus it appears that resorufin acetate may be just small enough to fit into the binding pocket, which is interesting in view of the critical specificity this site can exhibit, accepting tyrosine for instance, but not *p*-iodophenylalanine (*16*). It is also relevant here that proflavin has been used as a competitive inhibitor of chymotrypsin reactions (*17*); proflavin is quite similar in size, shape, and delocalized electronic structure to resorufin. In this work, no evidence of product inhibition by resorufin was seen, probably because resorufin is negatively charged whereas proflavin is positively charged and this difference is expected to result in markedly different tendencies to bind to chymotrypsin (*18*).

Figure 3 shows Lineweaver–Burk plots for the steady-state rate of hydrolysis of *p*-nitrophenyl acetate (A) and resorufin acetate (B) carried out under identical conditions. At high *p*-nitrophenyl acetate concentration, substrate activation is evident, as has previously been reported (*12, 19*). This implies that this substrate can bind in more than one mode to the enzyme, possibly involving the binding "subsites" [such as the S_1' or leaving group site (*13*)]. With resorufin acetate, solubility limitations preclude the use of the very high concentrations at which *p*-nitrophenyl acetate gives activation. From the back-projection of the linear part of Fig. 3A, and taking into account the enzyme concentration and the appropriate value of ε for *p*-nitrophenoxide at this pH (determined experimentally as 16,600), the turnover number is calculated to be 0.013 s^{-1}. The value of K_M is approximately 24 μM. [These data may be compared with values of 0.0056 s^{-1} and 1.59 μM obtained at pH 7.8 (*12*). Previous work would suggest that as the pH changes from 7.8 to 8.6 there would be little change in k_{cat} but an increase in K_M for chymotrypsin substrates,