BIOORGANIC CHEMISTRY 21, 354–365 (1993)

3,4-Dihydro-3-methyl-6-nitro-2*H*-1,3-benzoxazin-2-one, a Reagent for Labeling *p*-Nitrophenyl Esterases with a Chromophoric Reporter Group—Synthesis and Reaction with Chymotrypsin

TREVOR M. KITSON AND GRAHAM H. FREEMAN

Department of Chemistry and Biochemistry, Massey University, Palmerston North, New Zealand

Received May 17, 1993

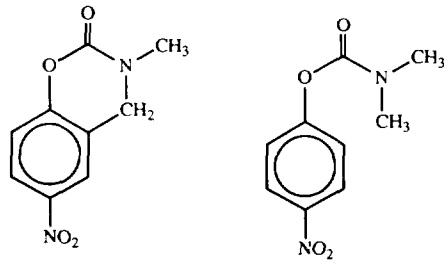
We report the synthesis of 3,4-dihydro-3-methyl-6-nitro-2*H*-1,3-benzoxazin-2-one (DMNB), a close structural analogue of *p*-nitrophenyl dimethylcarbamate. DMNB is unstable in aqueous solution when exposed to light, but is stable in the dark. The compound reacts slowly with chymotrypsin as an analogue of the substrate, *p*-nitrophenyl acetate, and this results in the incorporation of a *p*-nitrophenol-containing "reporter group" within the enzyme's active site. The pK_a of this group is two pH units higher than that of free *p*-nitrophenol, a result consistent with a hydrophobic environment around the enzymic binding site. Denaturation of the enzyme returns the pK_a to its unperturbed value. DMNB has the promise of being a useful active site-directed reagent for other enzymes with *p*-nitrophenyl esterase activity. © 1993 Academic Press, Inc.

INTRODUCTION

The concept of the "reporter group" was introduced by Burr and Koshland in 1964 (*1*). Essentially, the idea is that observation of some physical property of a chemical group that is covalently linked to a macromolecule can in principle provide useful information about the microenvironment of the bound group. For example, this technique might be used to probe the polarity of an enzyme's active site, or to monitor a change in the enzyme's three-dimensional structure when it interacts with a cofactor. Physical parameters that are potentially useful are light absorbance, fluorescence, acidity or basicity, or NMR or ESR spectrum. Some examples of the application of the concept are given in Refs. (*2–11*).

This paper describes the synthesis and properties of 3,4-dihydro-3-methyl-6-nitro-2*H*-1,3-benzoxazin-2-one (DMNB; Scheme 1). The closely related noncyclic analogue *p*-nitrophenyl dimethylcarbamate has been successfully used to identify the active site nucleophile of aldehyde dehydrogenase from sheep liver cytosol (*12*). The latter compound reacts with this enzyme by attaching a stable dimethylcarbamyl group to Cys-302, with the concomitant liberation of *p*-nitrophenoxide. It was reasoned that DMNB would undergo a similar chemical reaction but that the chromophoric product would remain covalently attached to the enzyme as a useful reporter group. Furthermore, this reaction potentially could happen with any of the large number of enzymes that catalyze the hydrolysis of *p*-nitrophenyl

0045-2068/93 $5.00



DMNB p-nitrophenyl dimethylcarbamate

SCHEME 1

esters. The acidity of the enzyme-linked *p*-nitrophenol group should be critically dependent on its environment within the active site of the enzyme under investigation, and this acidity is easily measurable since the *p*-nitrophenoxide ion (but not the un-ionized *p*-nitrophenol) absorbs in a convenient region of the spectrum (approximately 400 nm). Experiments to test these expectations, using chymotrypsin and trypsin as the enzymes, are described below.

MATERIALS AND METHODS

Preparation of DMNB

The synthetic route is shown in Fig. 1. Salicylaldehyde (2-hydroxybenzaldehyde, 36.8 g) was mixed slowly with methylamine (64 ml of a 25%, w/v, aqueous solution). Heat was evolved. The mixture was stirred overnight at room temperature. The product (**I**, *N*-methyl-2-hydroxybenzaldimine) was extracted into diethyl ether and subsequently purified by vacuum distillation to give an oily liquid (83%). δ ($CDCl_3$): 3.4 (s, 3H), 6.7–7.5 (m, 4H), 8.3 (s, 1H). Compound **I** (33.7 g) was dissolved in ethanol (50 ml) and cooled on ice. Sodium borohydride (9 g) was added over a period of 3 h, cooling on ice. Water was added, the amine (**II**, *N*-methyl-2-hydroxybenzylamine) was extracted into diethyl ether, and, after attempts at drying, the solvent was evaporated, giving an oily liquid. It proved impossible to remove all traces of water from the product, which was used in the next synthetic step without further purification. δ ($CDCl_3$): 2.5 (s, 3H), 4.0 (s, 2H), 6.1 (s, 2H, –OH, and –NH–), 6.7–7.4 (m, 4H). Compound **II** (6.14 g) was dissolved in toluene (50 ml) and *N*,*N*-dimethylaniline (12.5 ml) was added. Phosgene (carbonyl chloride, in toluene, 12.5%, w/w, 45 ml) was added over 30 min, cooling to 10°C. (Phosgene is of course highly toxic and should be used with care in an efficient fume hood.) The mixture was stirred overnight at room temperature and then refluxed for 30 min. The toluene solution was washed with HCl (2 M), dried, and evaporated. The product (**III**, 3,4-dihydro-3-methyl-2*H*-1,3-benzoxazin-2-one) was recrystallized from ethanol as white crystals (23%), mp 106–108°C. δ ($CDCl_3$):

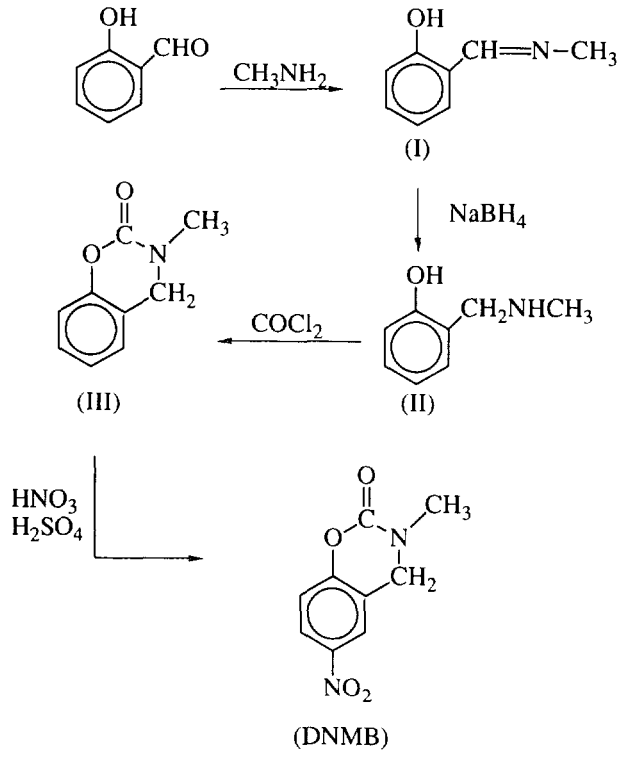


FIG. 1. Route used for the synthesis of DMNB.

3.2 (s, 3H), 4.6 (s, 2H), 6.9–7.4 (m, 4H); carbonyl peak at 1710 cm^{-1}. Compound **III** (1.7 g) was dissolved in acetic acid (3 ml) and concd. H_2SO_4 (4 ml) was added. The mixture was cooled to 0°C. A cooled mixture of concd. HNO_3 (0.8 ml) and concd. H_2SO_4 (1 ml) was then added slowly and the temperature was maintained below 10°C. The mixture was left at room temperature for 1.5 h and then poured onto ice (20 g). The product (DMNB) was extracted into chloroform and the solution was washed with sodium bicarbonate solution. Drying and evaporating the chloroform left a solid that was recrystallized from ethanol as colorless crystals (80%), mp 202–204.5°C. δ ($CDCl_3$): 3.17 (s, 3H), 4.58 (s, 2H), 7.08–7.23 (d, 1H), 8.10–8.26 (d, 1H), 8.10 (s, 1H, superimposed). $C_9H_8O_4N_2$ confirmed by mass spectrometry (*m/z* = 208.0484069).

6-Nitrodihydrocoumarin

This was prepared from dihydrocoumarin by a nitration under the same conditions as used above for DMNB. Recrystallization from acetone gave pale yellow crystals, 134–136°C. δ ($CDCl_3$): 2.6–3.4 (m, 4H), 7.15–7.32 (d, 1H), 8.1–8.4 (d, 1H and s, 1H, superimposed).

Isolation of DMNB-labeled chymotrypsin and trypsin

α-Chymotrypsin (Aldrich, type II, from bovine pancreas) was dissolved in 200 mM sodium phosphate buffer, pH 8.0 (3 ml) giving either an approximately 0.3 or 0.4 mM solution. DMNB in acetone (0.1 ml) was added to give a concentration of 0.6 or 0.8 mM, respectively. The mixture was protected from light and left at 25°C for 48 h. The solution was then passed down a column of Bio-Gel P-6 (25 × 0.8 cm), eluting with 10 mM sodium phosphate buffer, pH 7.4, and the labeled enzyme was collected in a volume of 7 ml well separated from excess modifer. Trypsin (Sigma, from bovine pancreas, treated with a chymotrypsin inhibitor) was treated with DMNB using the same procedure.

Denatured DMNB-labeled chymotrypsin

Labeled enzyme solution (7 ml), as prepared above, was mixed with perchloric acid (70 μl of a 70% w/v aqueous solution) and the protein that precipitated was collected by brief centrifugation. The precipitate was washed two or three times with water by dispersal with a glass rod, centrifugation, and decantation, and finally the precipitate was dissolved in 10 M aqueous urea (20 ml).

Determination of the pK_a of the p-Nitrophenol Group in DMNB-Labeled Chymotrypsin

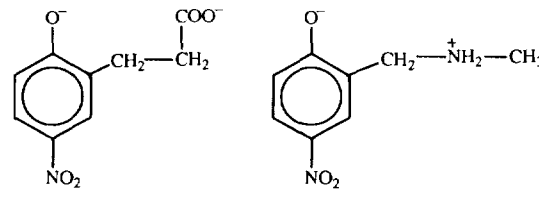
Samples of labeled enzyme (0.5 ml), prepared as above, were mixed with 1.5 ml of various buffers (*13*) and the absorbance spectrum from 300 to 550 nm was recorded at 20°C (unless otherwise stated) using a Varian Cary 1 spectrophotometer. With denatured chymotrypsin, 1-ml samples were mixed with 1 ml of the buffers. In measuring the absorbance of the *p*-nitrophenoxide peak, the absorbance at 550 nm was taken as zero. After recording the spectrum, the pH of each labeled enzyme sample was measured. The data were plotted using Enzfitter (*14*) to compute the best theoretical titration curve.

Enzyme Assay

The activities of chymotrypsin and trypsin were measured spectrophotometrically at 25°C in 200 mM sodium phosphate buffer, pH 8.0, using *p*-nitrophenyl acetate (0.1 mM) as substrate.

Light-Induced Hydrolysis of DMNB

DMNB in acetone (0.1 ml) was added to various buffers (3 ml) and the mixture was exposed to normal laboratory lighting conditions at 25°C. At intervals the absorption spectrum was recorded. When no further increase in absorption was observed, small samples of the solution were added to various buffers and the absorbance was determined as a function of pH.



(IV) (V)

SCHEME 2

RESULTS

Spontaneous Hydrolysis of DMNB

Early in this work it was found that aqueous solutions of DMNB are stable in the dark for several days, but fairly soon turn deep yellow when exposed to light. For example, at pH 7.0, DMNB (0.1 mM) is completely decomposed within less than 1 h, if not kept dark. (The rate of this reaction depends on the brightness of the lighting conditions). The λ_{max} of the product is 425–428 nm and its pK_a (determined from the variation of absorbance with pH) is 6.84. At pH 11.0, the hydrolysis is faster and the λ_{max} of the product is 409 nm. For comparison the λ_{max} values for the dianion of 3-(2-hydroxy-5-nitrophenyl)propanoic acid (**IV**; Scheme 2) (produced by alkaline hydrolysis of 6-nitrodihydrocoumarin) and the zwitterion of *N*-methyl-2-hydroxy-5-nitrobenzylamine (**V**) are 415 and 392 nm, respectively. The pK_a values for the phenol groups of these two compounds are 7.38 (this work) and 5.88 (*8*), respectively. *p*-Nitrophenol itself has a pK_a of 7.07 and its anion absorbs at 399 nm.

Effect of Solvents on the pK_a of p-Nitrophenol

We attempted to measure the pK_a of *p*-nitrophenol in the presence of various organic solvents and the results are shown in Table 1. While recognizing that the significance of pH values determined under such conditions is highly debatable (*15*), it is nevertheless probably reasonable to make the broad generalization that the pK_a increases as the medium becomes less polar.

Properties of DMNB-Modified Chymotrypsin

When chymotrypsin was incubated at 25°C and pH 8.0 with a twofold excess of DMNB, the activity of the enzyme (as measured with *p*-nitrophenyl acetate as substrate) slowly decreased. After 24 h, it was 44% of a control incubation without modifier, and after 46 h it was 27%.

After isolation of the modified enzyme as described above, the pK_a of the covalently linked *p*-nitrophenol group was determined, both before (9.03) and after (6.94) denaturation of the protein. The results are shown in Fig. 2. When attached to the unfolded protein, the pK_a is similar to that of free *p*-nitrophenol

TABLE 1

Acidity of *p*-Nitrophenol Determined in the Presence of Various Solvents

Solvent	pK_a
Water	7.1
1-Propanol (48%)	7.9
Acetone (58%)	8.2
Tetrahydrofuran (58%)	8.4
Ethanol (86%)	9.1

Note. Samples of *p*-nitrophenol were added to mixtures of various buffers and solvents at the concentrations shown, and the absorbance of the *p*-nitrophenoxide ion was measured. pK_a values were determined from plots of absorbance versus pH.

(7.07), but labeled protein that is presumably still in its native conformation shows a greatly increased pK_a value. The *p*-nitrophenoxide label absorbs at 395 nm in undenatured chymotrypsin, but this peak shifts to 414 nm upon denaturation. At low pH values, the undissociated *p*-nitrophenol group absorbs at 323 nm on the denatured enzyme; before unfolding, this peak is present at a lower wavelength as a shoulder on the protein absorbance.

The spectrum of a sample of DMNB-modified chymotrypsin at pH 8.5 was

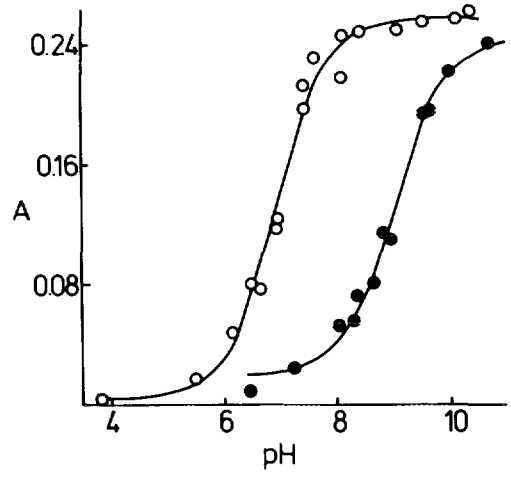


FIG. 2. Ionization profile of the reporter group in DMNB-modified chymotrypsin. The peak absorbance of the *p*-nitrophenoxide group is plotted against pH. ●, undenatured enzyme; ○, enzyme denatured by concentrated urea.

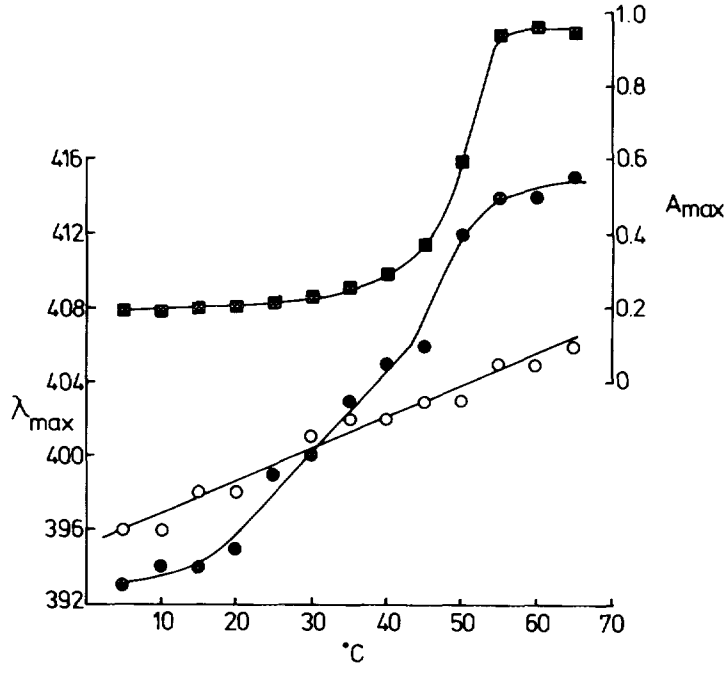


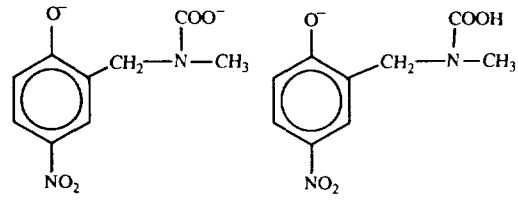
FIG. 3. Effect of temperature on the absorbance of DMNB-modified chymotrypsin. A sample of modified enzyme at pH 8.5 was subjected to increasing temperature and the λ_{max} (●) and maximal absorbance (■) of the reporter group were recorded. ○, change in λ_{max} of a control solution of unbound *p*-nitrophenoxide.

recorded at various temperatures and the results are shown in Fig. 3. The λ_{max} increases fairly steadily over the range 5 to 45°C (as does that of free *p*-nitrophenoxide). Between 45 and 55°C, λ_{max} shows a more pronounced rise, and over the same temperature range there is a very marked increase in the magnitude of the absorbance. The pK_a of the covalently linked *p*-nitrophenol group at 37°C is slightly lower than at 20°C (8.66 versus 9.03).

The ionization profile of free *p*-nitrophenol (20 μM) in the presence of chymotrypsin (100 μM) was determined. The λ_{max} value was found to be 397 nm and the pK_a to be 7.12, values virtually the same as those in the absence of chymotrypsin.

Properties of DMNB-Modified Trypsin

Under identical conditions (pH 8.0, 25°C, 0.1 mM substrate), trypsin was found to catalyze the hydrolysis of *p*-nitrophenyl acetate at 42% of the rate of chymotrypsin. However, trypsin was found to incorporate only 8% as much label from DMNB as chymotrypsin does under the conditions described above. It was still possible to determine the λ_{max} and pK_a values associated with the trypsin-linked label; the values are 406 nm and 6.4. (There will be appreciable experimental error in the latter figure as it depends on the measurement of relatively small absorbance values, especially at lower pH.)



(VI) (VII)

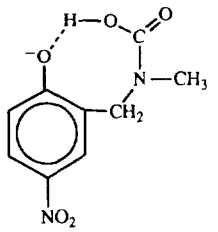
SCHEME 3

DISCUSSION

Light-Induced Hydrolysis of DMNB

Although the light-induced decomposition of DMNB is of no relevance to enzyme-labeling experiments carried out in the dark, we thought it would be of interest to consider what the product of the reaction may be.

At first sight, hydrolysis of DMNB might be expected to give the dianion (**VI**; Scheme 3) as the yellow product, but probably not the carbamic acid (**VII**). This is because carbamic acids are reportedly unstable (*16*), releasing carbon dioxide and giving the amine, which in this case would have structure (**V**) above at moderate pH values. However, we can eliminate (**V**) as a candidate for the product of the light-induced hydrolysis of DMNB as it has quite different values of λ_{max} and pK_a (see Results). (Clearly, the nearby positively charged amino group strongly affects the properties of the *p*-nitrophenol.) The product of the reaction at pH 7.0 is also unlikely to be (**VI**) as the structurally and electrically very similar species (**IV**) has values of λ_{max} and pK_a rather different from the experimentally determined values for the reaction product. For example, the pK_a of the reaction product is slightly less than that of *p*-nitrophenol, whereas that of the phenol which ionizes to give the model species (**IV**) is higher, as expected for a compound with a negatively charged side chain. We suggest that (**VI**) is in fact the product of the light-induced reaction at pH 11.0 (where λ_{max} is 409 nm, fairly close to that of the model species (**IV**), 415 nm), whereas the un-ionized carbamic acid (**VII**) is produced at pH 7.0. A carboxylic acid would of course be ionized at pH 7.0, but the electron-donating nitrogen atom of a carbamic acid would be expected to cause a comparatively high pK_a value for the –COOH group. It could be speculated that intramolecular hydrogen bonding as in structure (**VIII**; Scheme 4) (although involving an unusual eight-membered ring) may stabilize the carbamic acid group and perturb the absorbance of the *p*-nitrophenoxide ion to the unexpectedly long wavelength of 425–428 nm. Whether or not (**VIII**) is a product of the decomposition of DMNB, we are unsure at this stage as to how light is involved in the mechanism of the reaction. It is interesting to note that the noncyclic analogue of DMNB, *p*-nitrophenyl dimethylcarbamate, is perfectly stable in aqueous solutions in the light.



(VIII)

SCHEME 4

Environment of the Binding Site in Chymotrypsin

The loss of esterase activity as chymotrypsin reacts with DMNB is consistent with the expected reaction at the enzyme's active site. It is highly likely that DMNB carbamylates the enzyme's Ser-195 residue, just as *p*-nitrophenyl acetate acetylates it, but in the former case the rate of hydrolysis of the modified enzyme is evidently zero. As Fig. 4 makes clear, the label introduced by DMNB is of precisely the right size and character to mimic a typical acyl-enzyme involved in chymotrypsin-catalyzed peptide hydrolysis. It is also clear that covalent attach-

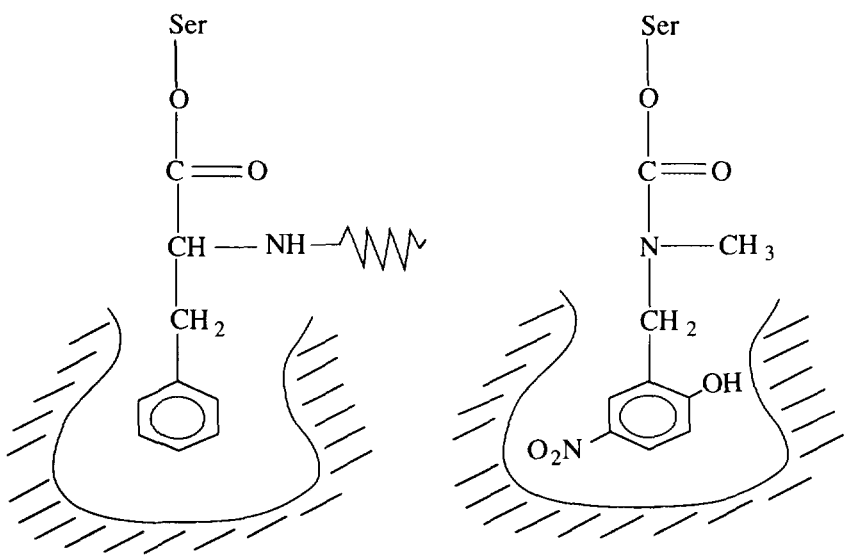


FIG. 4. Comparison of the DMNB-derived reporter group with part of the structure of a typical acyl-chymotrypsin intermediate. The drawing on the left shows schematically the intermediate in chymotrypsin-catalyzed peptide hydrolysis at a Phe residue, with the aromatic group bound in the enzyme's hydrophobic pocket. The drawing on the right shows the equivalent structure of DMNB-modified chymotrypsin.

ment of the reporter group is necessary, since chymotrypsin has no effect on the spectrum or acidity of unlinked *p*-nitrophenol (see Results).

What then does the reporter group "report" about the binding site of chymotrypsin? It tells us that the environment is one in which the *p*-nitrophenol group is markedly less acidic (by a factor of approximately 100) than free *p*-nitrophenol in aqueous solution. The peculiar environment is destroyed upon unfolding the protein by the influence of urea (Fig. 2) or heat (Fig. 3). In Fig. 3, the large increase in absorbance above 45–50°C is explained by a downward shift in pK_a upon heat denaturation. Intuitively, one would assume that the charged *p*-nitrophenoxide ion would be disfavored by a nonpolar environment relative to the neutral *p*-nitrophenol form. This is borne out by the observed increase in pK_a for *p*-nitrophenol in the presence of organic solvents (Table 1). [Similarly, the pK_a of acetic acid changes from 4.76 to 10.32 as the ethanol content of the solvent changes from 0 to 100% (*15*).] The blue shift of the λ_{max} of chymotrypsin-linked un-ionized *p*-nitrophenol (see Results) is also consistent with a nonpolar binding site (*2*).

It has of course long been known that chymotrypsin has a hydrophobic binding pocket that snugly fits around the amino acid side-chains for which the enzyme has a specificity (such as Phe, Tyr, and Trp). The present results are therefore not unexpected, but they show the potential value of DMNB as a reagent for less well-investigated enzymes. Even for chymotrypsin, previous experiments with reporter groups have given somewhat conflicting results. Thus, Kallos and Avatis (*2*) interpreted the spectral properties of a *p*-nitrobenzenesulfonyl group attached to chymotrypsin's active site Ser-195 in terms of a hydrophobic environment. Similarly, Sigman *et al.* (*5*) alkylated chymotrypsin at the nearby Met-192 using various phenacyl bromides and concluded from absorption spectra of model compounds that the environment of this site is less polar than water. On the other hand, Hille and Koshland (*3*) found that 2-bromoacetamido-4-nitrophenol labels Met-192 and "reports" an environment that is *more* polar than water. Clearly, the DMNB-derived reporter group used here is particularly significant in this sort of study since it so elegantly resembles a true acyl-chymotrypsin intermediate (Fig. 4).

DMNB and Trypsin

It is perhaps not surprising that a neutral aromatic compound such as DMNB reacts much better with chymotrypsin than with trypsin (which is "designed" for positively charged amino acid residues such as Arg and Lys). The relatively small amount of label incorporated into trypsin during a 2-day incubation has a pK_a (bearing in mind the likely error in this value) not very far removed from that of free *p*-nitrophenol. If the label were actually in the trypsin binding pocket one would expect the negatively charged Asp-189 residue (which controls trypsin specificity) to disfavor the negative *p*-nitrophenoxide group—that is, to raise the pK_a considerably above 7. Thus one might speculate that after reaction between trypsin and DMNB, the covalently linked *p*-nitrophenoxide group is electrostatically expelled from the binding pocket by the Asp residue, so that the label protrudes into the surrounding aqueous solvent.

Potential for DMNB as a Reagent for Other p-Nitrophenyl Esterases

The following points are relevant here. (1) DMNB is only marginally larger than *p*-nitrophenyl acetate, so steric hindrance to its reaction with enzymes that have a *p*-nitrophenyl esterase capacity would probably be insignificant. (2) DMNB is inherently rather unreactive (apart from its light-induced hydrolysis) and thus nonspecific reaction with various functional groups on an enzyme's surface would probably be insignificant, compared to its specific reaction at the esterase active site. Some other reagents that have been used to supply reporter groups are likely to be more indiscriminately reactive (*4, 7, 9, 10*). (3) After reaction of DMNB with an enzyme, the resulting enzyme-linked carbamyl group is even less reactive. The nitrogen atom enormously reduces the electrophilicity of the adjacent carbonyl group and so there is every likelihood of the acyl-enzyme being essentially inert (as shown here with chymotrypsin). Contrast this with the use of 6-nitrodihydrocoumarin as a substrate for chymotrypsin (*17*). This compound may be thought of as introducing a transient reporter group, but hydrolysis of the acyl-enzyme follows rapidly upon the acylation step, as the acyl-enzyme is an ordinary ester (not a carbamate as it is with DMNB). (4) The *p*-nitrophenol group that DMNB connects to an enzyme's active site has a pK_a that is within the pH range of convenience and interest as regards most enzymes and is very sensitive to the group's environment. (5) The stable chromophoric label that DMNB introduces can potentially be used to identify an enzyme's catalytically essential group in sequencing studies. (6) Unpublished preliminary experiments with cytoplasmic aldehyde dehydrogenase show that DMNB reacts with this enzyme (faster than with chymotrypsin) to give a label whose pK_a is dramatically altered upon binding of the enzyme's cofactor, NAD^+, but not the reduced form, NADH. This enables many interesting experiments to be planned.

With these considerations in mind, it seems very likely that DMNB will prove to be a valuable versatile reagent for investigating the active sites of a variety of enzymes, combining as it does the concept of the reporter group with that of the active site-directed, mechanism-based inhibitor.

ACKNOWLEDGMENT

We are very grateful to the New Zealand Lottery Grants Board for funding for the spectrophotometer used in this work.

REFERENCES

1. BURR, M., AND KOSHLAND, D. E. (1964) *Proc. Natl. Acad. Sci. USA* **52,** 1017–1024.
2. KALLOS, J., AND AVATIS, K. (1966) *Biochemistry* **5,** 1979–1983.
3. HILLE, M. B., AND KOSHLAND, D. E. (1967). *J. Am. Chem., Soc.* **89,** 5945–5951.
4. MCMURRAY, C. H. AND TRENTHAM, D. R. (1969). *Biochem. J.* **115,** 913–921.
5. SIGMAN, D. S., TORCHIA, D. A., AND BLOUT, E. R. (1969) *Biochemistry* **8,** 4560–4566.
6. FURLANETTO, R. W., AND KAISER, E. T. (1970) *J. Am. Chem. Soc.* **92,** 6980–6982.
7. FOLK, J. E., AND GROSS, M. (1971) *J. Biol. Chem.* **246,** 6683–6691.

8. FREY, P. A., KOKESH, F. C., AND WESTHEIMER, F. H. (1971) *J. Am. Chem. Soc.* **93,** 7266–7269.
9. MARSHALL, M., AND COHEN, P. P. (1980) *J. Biol. Chem.* **255,** 7296–7300.
10. WOHLFEIL, E. R., AND HUDSON, R. A. (1991) *Biochemistry* **30,** 7231–7241.
11. TRITSCH, D., MUNCH, O., AND BIELLMAN, J. F. (1990) *Bioorg. Chem.* **18,** 91–106.
12. KITSON, T. M., HILL, J. P., AND MIDWINTER, G. G. (1991) *Biochem. J.* **275,** 207–210.
13. MCKENZIE, H. A. (1969) *in* Data for Biochemical Research (Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M., Eds.), pp. 475–508, Oxford University Press, London.
14. LEATHERBARROW, R. J. (1987) *in* Enzfitter Manual, Elsevier Science Publishers BV, Amsterdam.
15. BATES, R. G. (1954) Determination of pH—Theory and Practice, p. 195, Wiley, New York/London/Sydney.
16. SMITH, P. A. S. (1965). Open-Chain Nitrogen Compounds, Vol. 1, p. 263, Benjamin, New York.
17. KAISER, E. T. (1970) *Acc. Chem. Res.* **3,** 145–151.