Photoaffinity labelling of carnitine acetyltransferase with *S*-(*p*-azidophenacyl)thiocarnitine

J. Matthew MAURO, Randolph V. LEWIS and Roland E. BARDEN*

Department of Chemistry, University of Wyoming, Laramie, WY 82071, U.S.A.

A photolabile reagent, *p*-azidophenacyl-DL-thiocarnitine, was synthesized and tested as a photoaffinity label for carnitine acetyltransferase (EC 2.3.1.7) from pigeon breast. *p*-Azidophenacyl-DL-thiocarnitine is an active-site-directed reagent for this acetyltransferase, since it is a competitive inhibitor (K_i 10 μ M) versus carnitine. U.v. irradiation of a mixture of *p*-azidophenacyl-DL-thiocarnitine and enzyme produces irreversible inhibition. Acetyl-DL-carnitine protects the enzyme from inhibition by photoactivated *p*-azidophenacyl-DL-thiocarnitine. In the presence of 30 mM-2-mercaptoethanol as a scavenger, the relationship between loss of activity and photoincorporation of reagent suggests that one molecule of reagent is incorporated per molecule of inhibited enzyme. However, peptide maps of enzyme labelled with *p*-azidophenacyl-[14 C]thiocarnitine indicate that several (about six) tryptic peptides (of a possible 60–65) are modified. The presence of 5 mM-acetyl-DL-carnitine significantly decreases the incorporation of reagent in each labelled tryptic peptide.

INTRODUCTION

L-Carnitine serves as a carrier of acyl groups in several enzymic and transport processes, related for the most part to the translocation of fatty acids across membranes (cf. Frenkel & McGarry, 1980). Structural analogues of carnitine esters that are capable of covalently labelling an acylcarnitine-binding site should be useful for characterizing structural aspects of several enzymes and transport proteins. Thus we have initiated a programme to develop photoaffinity probes based on acylcarnitines. The present work describes the synthesis of *S*-(*p*-azidophenacyl)-DL-thiocarnitine and initial experiments testing the new reagent as a photoaffinity probe for the acylcarnitine-binding site of CAT.

CAT (EC 2.3.1.7) catalyses the following reversible reaction:

In the years since CAT first became available in highly purified form from pigeon breast-muscle homogenate (Chase *et al.*, 1965), three studies employing active-site-directed irreversible inhibitors (affinity labels) have been conducted with the enzyme. Chase & Tubbs (1970) used bromoacetyl-L-carnitine to inactivate CAT, and the reagent specifically modified a histidine side chain present in the active site. More recently, thiol-specific affinity labels that are analogues of CoA have been used to inactivate CAT, presumably by modification of a cysteine residue (or residues) in or near the CoA-binding site on the enzyme (Nishimura *et al.*, 1982; Venkatraghavan & Smith, 1983).

The affinity-labelling reagents used in the studies cited above depend upon the presence of a nucleophilic residue in or near the substrate-binding site in order for irreversible modification of the enzyme to be successful.

A more attractive approach is the photoaffinity-labelling technique, which relies on a highly reactive intermediate that is formed upon irradiation of a photolabile substrate analogue. Owing to the very reactive nature of the photogenerated intermediate, covalent incorporation of the analogue into the binding site may be achieved even in the absence of a properly aligned nucleophilic amino acid residue (Bayley & Knowles, 1977). Thus photoaffinity probes provide a more reactive and less selective reagent with which to explore the structure of substrate-binding sites on enzymes, while using the basic affinity-labelling strategy.

A preliminary report of some experiments reported here has appeared (Mauro *et al.*, 1983).

EXPERIMENTAL

Materials

Chemicals were obtained from the following sources: *p*-azidophenacyl bromide from Pierce Chemical Co.; DL-[carboxy- 14 C]carnitine hydrochloride from New England Nuclear; DL-carnitine hydrochloride, 2-mercaptoethanol and thioacetic acid from Aldrich Chemical Co.; L-carnitine hydrochloride and CoA (lithium salt) from P-L Biochemicals; CAT (from pigeon breast muscle, catalogue no. C-8757, lot no. 121F-0373) from Sigma Chemical Co.; trypsin (EC 3.4.4.4) [tosylphenylalanyl-chloromethane ('TPCK')-treated; 217 units/mg] from Worthington Biochemical Corp.; an electrophoresis standard M_r -marker kit (MW-SDS-200) from Sigma Chemical Co.; acrylamide and *NN'*-methylenebisacrylamide (electrophoresis grade) were purchased from Eastman. All other chemicals and reagents were of the highest grade available from commercial sources.

The following compounds were synthesized by the indicated published method. DL-Acetylcarnitine was

Abbreviations used: CAT, carnitine acetyltransferase; PAP-TC, *S*-(*p*-azidophenacyl)thiocarnitine.

* To whom correspondence should be addressed.

synthesized by the method of Ziegler *et al.* (1967), DL-acetylthiocarnitine was synthesized by the method of Duhr *et al.* (1983), and acetyl-CoA was prepared by the method of Simon & Shemin (1953).

Cellulose t.l.c. plates (no. 5502, plastic-backed) and F₂₅₄ silica-gel t.l.c. plates (no. 5775-7, plastic-backed) were obtained from E. Merck. Aquasol-2 universal scintillation cocktail was purchased from New England Nuclear.

Methods

PAP-TC was prepared by coupling DL-thiocarnitine (Duhr *et al.*, 1983) with the photolabile ligand *p*-azidophenacyl bromide (Hixson & Hixson, 1975). [We are now aware that a synthesis of DL-thiocarnitine was first described in a patent awarded to Sigma-Tau Industrie Farmaceutiche Riunite (1982).] In a typical preparation, DL-acetylthiocarnitine hydrochloride (50 mg, 195 μ mol) was hydrolysed for 2 min with 3.5 ml of 0.4 M-KOH (N₂-purged), then neutralized to pH 8 with 1 M-HCl. This solution was immediately added to a rapidly stirred solution prepared by dissolving *p*-azidophenacyl bromide (48 mg, 200 μ mol) in 15 ml of propan-2-ol. To the resulting solution 2 ml of freshly prepared 1 M-KHCO₃ was added, and stirring was continued at room temperature under dim light. A few drops of the reaction mixture were removed and tested for the presence of thiol in a buffered solution, pH 7.5, containing 0.1 mM-5,5'-dithiobis-(2-nitrobenzoic acid). When the test showed no remaining free thiol groups, the solution was acidified to approx. pH 2.8 with 1 M-HCl, and the solvent was removed by rotary evaporation at 30 °C. The residue obtained was dried in a vacuum desiccator over P₂O₅ in the dark overnight, then extracted with three 10 ml portions of dry methanol. To the pooled methanolic extracts, anhydrous diethyl ether was added to precipitate PAP-TC. The solid material was washed with ether, dried briefly with a stream of N₂, then redissolved in methanol and reprecipitated with ether. The final product was dried over P₂O₅ and stored in the dark at 4 °C. The product prepared in this way was contaminated with small amounts of KCl and thiocarnitine disulphide. Aqueous solutions of PAP-TC (approx. pH 7) were observed to be stable indefinitely when stored frozen at -20 °C in the dark. Yield, 90%; u.v. λ_{max} (in methanol), 292 nm; u.v. λ_{max} (in water) 299 nm, $\epsilon = 20\,100\text{ M}^{-1}\cdot\text{cm}^{-1}$; i.r. (KBr disc), 2160 cm⁻¹ (-N₃); ¹H n.m.r. (²H₂O) (see Duhr *et al.*, 1983), δ 2.92 (2H), 3.30 (9H), 3.62 (1H), 3.71 (2H), 4.28 (2H), 7.18 (aromatic, doublet, 2H) and 8.05 p.p.m. (aromatic, doublet, 2H); t.l.c. (silica gel) (chloroform/methanol/water/formic acid, 25:21:4:1, by vol.), *R_F* 0.35 (PAP-TC) and *R_F* 0.03 (thiocarnitine disulphide); t.l.c. (cellulose) (butan-1-ol/acetic acid/water, 5:2:3, by vol.), *R_F* 0.83 (PAP-TC) and *R_F* 0.48 (thiocarnitine disulphide). For analytical work t.l.c. plates containing a fluorescent indicator were used, and PAP-TC was detected with a UVS-11 Mineralight lamp. Absorption spectra were obtained with an Hitachi 100-80 u.v.-visible spectrophotometer. N.m.r. spectra were obtained with a JEOL FX 270 MHz spectrometer.

Final purification to remove thiocarnitine disulphide (approx. 5% impurity) and KCl was accomplished by preparative t.l.c. Impure PAP-TC (5-10 mg) was dissolved in 0.5 ml of anhydrous methanol, and the solution was streaked on to a 20 cm $\times$ 20 cm cellulose-coated t.l.c.

plate (without fluorescent indicator). The plate was developed in the dark in butan-1-ol/acetic acid/water (5:2:3, by vol.), and dried in a vacuum desiccator over KOH pellets for 24 h. The PAP-TC was located by cutting a few thin guide strips from the plate and exposing them to I₂ vapour. The cellulose band containing PAP-TC was scraped free and eluted with two 0.6 ml portions of water; the cellulose was removed by centrifugation and the supernatant, containing purified PAP-TC, was removed and stored frozen at -20 °C in the dark. All PAP-TC used in enzyme experiments was purified in this way.

Synthesis of PAP-[¹⁴C]TC was carried out on a small scale with DL-[carboxy-¹⁴C]carnitine hydrochloride (approx. 10 μ Ci/ μ mol) as the source of radioactive label. DL-Acetyl[carboxy-¹⁴C]thiocarnitine was prepared by using the method described by Duhr *et al.* (1983), except that all quantities were greatly decreased in order to avoid excessive dilution of radioactivity. The small amount of DL-acetyl[carboxy-¹⁴C]thiocarnitine intermediate prepared was not isolated in pure form. Instead, base hydrolysis and subsequent coupling to *p*-azidophenacyl bromide was conducted, and purification by t.l.c. as described above was performed to allow isolation of PAP-[carboxy-¹⁴C]TC. Yield, 25%, based on starting DL-[carboxy-¹⁴C]carnitine hydrochloride; t.l.c. analysis of the purified material in either solvent system described above gave a single radioactive spot that coincided with authentic PAP-TC; specific radioactivity was 9.8 μ Ci/ μ mol.

The photolability of PAP-TC was demonstrated by irradiating a buffered solution (pH 7.8) of the analogue (approx. 20 μ M) with a UVL-21 Blak-Ray lamp (for which the wavelength of maximum intensity is 366 nm). The solution was contained in an unstirred quartz cuvette placed 0.5 cm from the light-source. Sequential u.v. spectra were recorded after each timed irradiation period to monitor photodecomposition. Another test of photolability demonstrated that the analogue was photoincorporated into cellulose t.l.c. plates upon irradiation at 366 nm, following a procedure described by Lau *et al.* (1977).

CAT was assayed by two different methods. In the first method, release of CoA was measured by monitoring the change of absorbance at 412 nm in the presence of 5,5'-dithiobis-(2-nitrobenzoic acid) (Bergmeyer, 1974). Assays were performed at 30 °C with a Gilford model 240 recording spectrophotometer equipped with constant-temperature circulating bath. For assay of photolabelled solutions of CAT and PAP-TC, a typical assay cuvette contained, in a volume of 1.0 ml, approx. 0.5 μ g of enzyme, 125 μ mol of Tris/HCl buffer, pH 7.8, 1.0 μ mol of L-carnitine, 150 nmol of acetyl-CoA, 120 nmol of 5,5'-dithiobis-(2-nitrobenzoic acid) and 500 nmol of EDTA. In reversible-inhibition experiments with PAP-TC, the above assay system was also used with the exceptions that each assay cuvette containing 250 nmol of acetyl-CoA and various amounts of L-carnitine (50 to 400 nmol) and PAP-TC (10, 50 or 100 nmol). A second assay system was employed when determining the remaining CAT activity in the photoinhibition solutions containing 2-mercaptoethanol. This system monitored the formation of the thioester bond of acetyl-CoA at 232 nm (Chase, 1965). A typical assay cuvette contained, in 1.0 ml, approx. 0.5 μ g of enzyme, 125 μ mol of Tris/HCl buffer, pH 7.8, 2.5 μ mol of DL-acetylcarnitine,

200 nmol of CoA and 500 nmol of EDTA. Reactions were started by the addition of enzyme.

For photoinhibition experiments, CAT [received from Sigma as a 3.2 M-(NH₄)₂SO₄ suspension] was dialysed into 375 mM-Tris/HCl buffer, pH 7.8, at 4 °C so that the concentration of (NH₄)₂SO₄ was reduced to less than 1 μM. Photoinhibition of CAT was performed in solutions (250 μl) containing approx. 10 μg of dialysed CAT, the appropriate amount of PAP-TC delivered from a stock solution, water, and Tris/HCl buffer, pH 7.8, to a final concentration of 125 mM. Solutions were made up in individual wells of an ice-cooled Pyrex spot plate equipped with magnetic-‘flea’ stirrers. At 1 min after addition of the enzyme, solutions were irradiated for 4.0 min with a UVL-21 Blak-Ray lamp positioned 0.5 cm from the sample. Immediately after the irradiation period, duplicate 10 μl portions were removed for assay of remaining enzyme activity. Control experiments without PAP-TC and with pre-irradiated PAP-TC were conducted. In experiments demonstrating protection of the CAT active site by DL-acetylcarnitine, the ester was added to a final concentration of 0.5–1.0 mM and the above protocol was followed.

In preparing CAT for photoinhibition with PAP-TC in the presence of 30 mM-2-mercaptoethanol, the enzyme was dialysed as noted above except that 75 mM-2-mercaptoethanol was present in the dialysis medium. Final dilution of the enzyme into the photoincubation solutions brought the concentration of 2-mercaptoethanol to 30 mM.

To measure photoincorporation of PAP-[¹⁴C]TC into CAT upon photolysis, photolysed solutions were removed into small dialysis bags and exhaustive dialysis was carried out for a total of 48 h against four 1-litre changes of 150 mM-Tris/HCl buffer, pH 7.5. After dialysis, separate samples were removed for protein assay and scintillation counting of radioactivity in 10 ml of Aquasol-2. PAP-[¹⁴C]TC of known specific radioactivity was used to calibrate the scintillation-counting system under the conditions of these experiments.

SDS/polyacrylamide-slab-gel electrophoresis was performed with 8% acrylamide gels (Owens & Haley, 1976). Fixed gels were stained with Coomassie Brilliant Blue, destained and dried on to Whatman 3MM paper by using a Hoefer slab-gel drier. Radioactive gels were treated with NEN En³Hance autofluorography enhancer, according to the manufacturer's directions, before drying (60–70 °C for 2 h) and autofluorography. Autofluorography was performed at –70 °C for 5–10 days with Kodak X-OMAT-AR X-ray film.

CAT (2 mg) was prepared for amino acid analysis by S-carboxymethylation in 7 M-guanidinium chloride/dithiothreitol solution (Crestfield *et al.*, 1963) by using a modified procedure as described by Koide *et al.* (1978). After S-carboxymethylation, excess reagents were removed by dialysis against 50 mM-Tris/HCl buffer, pH 8.6. Dialysis resulted in precipitation of at least 90% of the protein. A portion of the precipitated protein (approx. 500 μg) was redissolved in 2 M-urea, then dialysed extensively against distilled water. The precipitated protein was hydrolysed at 110 °C with 6 M-HCl/1% thioglycolic acid in a sealed evacuated tube for 24 h. Pre-column modification with *o*-phthalaldehyde was carried out by using the method of Jones *et al.* (1981); separation and quantification of the modified amino acids was carried out by using an Alltech C₁₈ reverse-phase

h.p.l.c. column. Calculation of mol of individual amino acids per mol of enzyme was based on an *M_r* of 55000 for CAT (Chase & Tubbs, 1969).

CAT (550 μg/ml) was prepared for trypsin digestion by photoinhibition with 20 μM-PAP-[¹⁴C]TC (9.8 μCi/μmol) in the presence of 10 mM-2-mercaptoethanol and 50 mM-Tris/HCl buffer, pH 7.8. After removal of unbound photolysis products by extensive dialysis, first against 50 mM-Tris/HCl buffer, pH 7.8, containing 5 mM-DL-carnitine and 0.5 M-NaCl and then against 50 mM-Tris/HCl buffer, pH 7.8, the inhibited enzyme was treated sequentially (for 4 h at 37 °C) with two 1% (w/w) portions of trypsin (217 units/mg). An identical experiment, performed as a control, had 5 mM-DL-acetylcarnitine present in the photolysis solution as an active-site protector.

Peptide fragments obtained from trypsin digestion were subjected to reverse-phase h.p.l.c. analysis in order to obtain low-resolution peptide maps for the digested PAP-[¹⁴C]TC-modified protein. The C₁₈ h.p.l.c. system (Lewis, 1979) was used with 0.5 M-acetate/0.2 M-pyridine buffer, pH 4.0, with propan-1-ol as the organic modifier. The column eluent was monitored with a post-column fluorescence detection system (Bohlen *et al.*, 1975). Other details of the separation procedure are described in the legend to Fig. 3. Effluent from the h.p.l.c. column was collected in about 60 1.25 ml fractions in 3.0 ml polypropylene tubes. Fractions were evaporated to a volume of approx. 100 μl each in a Savant centrifugal evaporator, then 2.5 ml of distilled water was added to each tube and the evaporation to a small volume (approx. 100 μl) was repeated. One more cycle of this co-evaporation step was then performed to ensure removal of pyridinium acetate buffer salt, which, if present, interfered with subsequent scintillation counting. The final contents of each tube (approx. 200 μl) were transferred quantitatively to separate scintillation vials and their radioactivities counted in 5 ml of Aquasol-2. Scintillation counting was performed with a Beckman LS-7500 microprocessor-controlled scintillation counter.

(–)-Carnitine and acetyl-(–)-carnitine were assayed by the method of Bergmeyer (1974). Steady-state kinetic data were evaluated by using the COMPO program of Cleland (1967). Protein concentrations were determined principally by the dye-binding method of Bradford (1976), with bovine serum albumin as the protein standard. With CAT, the method of Lowry *et al.* (1951) gave results identical with those obtained by the dye-binding method.

RESULTS AND DISCUSSION

Photodecomposition of PAP-TC

Irradiation of PAP-TC in buffered solutions resulted in smooth decomposition of the aryl azide function (Fig. 1). Light-induced decomposition was 97% complete after 3 min of irradiation under the conditions of these experiments. The presence of two isosbestic points (Fig. 1) suggests that PAP-TC photodecomposes to a single type of u.v.-absorbing product. Total light-absorption measured at 299 nm decreased to 35% of the value of unphotolysed samples, indicating that extensive decay of the aryl azide chromophore had taken place as a result of irradiation. The azide function of PAP-TC was stable

Fig. 1. Absorption spectra of PAP-TC after irradiation for fixed lengths of time

Samples ($25 \mu\text{M}$) were dissolved in 0.5 M-potassium phosphate buffer, pH 7.5. The lengths of irradiation (in min) are shown on the spectra.

to reduction for at least 10 min when incubated in the presence of 30 mM-2-mercaptoethanol at pH 7.8, as judged by the lack of any change in the absorption spectrum in the 280–320 nm range. When irradiated in the presence of 2-mercaptoethanol, the reagent photo-decomposed as described above (results not shown).

Reversible inhibition of CAT by PAP-TC

Steady-state kinetics experiments (no photolysis) showed that PAP-TC is a linear competitive inhibitor versus L-carnitine (Fig. 2). Thus PAP-TC and L-carnitine appear to compete for the same binding site on the enzyme (alternative interpretations of linear competitive inhibition cannot be excluded), and PAP-TC can be described as an active-site-directed reagent. The computed K_i value of $10 \pm 3 \mu\text{M}$ was unexpectedly low. If the measured K_m values are proportional to the true dissociation constants, then the binding site has a considerably greater affinity for PAP-TC than for either L-carnitine (K_m 120 μM) or acetyl-L-carnitine (K_m 350 μM) (Chase, 1967). We are not aware of previous published studies in which an aromatic acyl group (or 'pseudo' acyl group, such as in PAP-TC) has been substituted in place of the normal aliphatic ester group on carnitine and then tested as an inhibitor for CAT. Therefore we do not know whether the unusually tight binding is unique to the aryl

Fig. 2. Reversible inhibition of CAT by unphotolysed PAP-TC

The concentrations of PAP-TC tested are shown on the graph (in μM). The unit of velocity, v , is $\Delta A_{412}/\text{min}$. Other experimental details are given in the Experimental section. Inset: secondary plot of slopes ($\times 10$) versus PAP-TC concentration.

azide photoprobe, or a general property of aryl esters of carnitine.

Photoinhibition of CAT by PAP-TC

Photolysis at 0°C of incubating solutions of CAT containing increasing concentrations of the photoaffinity probe (10–40 μM) resulted in increasing amounts of irreversible inhibition up to 40 μM -PAP-TC, where 54% inhibition occurred (Mauro, 1983). Increasing the concentration of PAP-TC beyond 40 μM resulted in little increase in inhibition. The addition of acetyl-DL-carnitine to the photolysis medium resulted in partial protection of the enzyme from inhibition by photoactivated PAP-TC. For example, in the system where photolysis of 40 μM -PAP-TC resulted in 54% inhibition, addition of 0.5 mM-acetyl-DL-carnitine decreased the inhibition to 37% and 1.0 mM of the protective substrate decreased it to 24%. Control experiments showed that PAP-TC did not irreversibly inhibit CAT in the absence of photolysis, nor was the enzyme inhibited in the absence of reagent under the photolysis conditions employed.

Photoinhibition was carried out in 125 mM-Tris/HCl buffer, pH 7.8, as well as in 125 mM-potassium phosphate buffer, pH 7.8, with nearly identical results (not shown). Bayley & Knowles (1977) have suggested that the presence of an organic buffer such as Tris can lead to anomalous photoinhibition results; our similar results obtained with two different buffers demonstrated that buffer effects were not important in the present experiments.

Although the above studies demonstrated that photoinactivation occurred in the expected manner and that acetyl-DL-carnitine provided site protection, a significant amount of photoinactivation was observed in the presence of high concentrations of the substrate. This

observation suggested that some photolabelling was occurring at loci outside of the active site. Our observation that PAP-TC is stable in the presence of fairly high concentrations of 2-mercaptoethanol (10–30 mM) for brief periods of time allowed experiments to be performed that tested for the effect of 'pseudo-photoaffinity labelling' (Ruoho *et al.*, 1973) and/or the effects of random covalent modification. In photoaffinity labelling, the ideal mechanism of inhibition occurs when the activated photoprobe is covalently incorporated into the binding site before diffusing out of the site. Ideally, any highly reactive photoactivated nitrene species generated outside the binding site should react preferentially with the relatively high concentrations of water and/or buffer components present before encountering an enzyme molecule, resulting in very little non-specific labelling. In 'pseudo-photoaffinity labelling', probe nitrenes generated outside the binding site inactivate the enzyme by diffusion into the binding site. It is evident that such a process is essentially a case of classical electrophilic affinity labelling where the classical electrophile has been replaced by a nitrene electrophile. The advantage gained by using a photoaffinity probe is lost in such cases (Bayley & Knowles, 1977).

A high concentration of a thiol compound acting as a nitrene scavenger (Maassen & Moller, 1975) should rapidly inactivate any highly reactive species generated outside the PAP-TC-binding site, eliminating inhibition due to the 'pseudo-photoaffinity-labelling' process described above, as well as reducing non-specific (random) labelling effects. When photoinhibition experiments were carried out as before except in the presence of 30 mM-2-mercaptoethanol, photoinduced irreversible inhibition of CAT was again observed (Table 1). The total inhibition observed at 40 μ M-PAP-TC was approx. 40% of the initial activity. Up to 54% inhibition had been observed in the absence of the scavenger (Mauro, 1983). The enzyme was significantly protected from inhibition by the presence of acetyl-DL-carnitine (Table 1). Since a substantial amount of photoinhibition occurred in the presence of a high concentration of 2-mercaptoethanol, the photoincorporated analogue very probably reacts within the binding site before diffusing into the bulk medium. The fact that the presence of the scavenger measurably reduced the degree of observed photoinhibition indicated that at least part of the original inhibition occurred by either the 'pseudo-photoaffinity-labelling' process or by random covalent attachment to the enzyme. Non-specific incorporation due to random bimolecular collisions between receptor proteins and apparently

relatively long-lived nitrene species generated from azide-based photoprobes has been a serious problem in several reported cases (Hixson & Hixson, 1973; Bayley & Knowles, 1978). In order to keep undesirable side reactions to a minimum, subsequent photolabelling of CAT with PAP-[14 C]TC was conducted with 2-mercaptoethanol (10–30 mM) present in all photoincubation solutions.

Previously published work, in which bromoacetyl-L-carnitine was used as an affinity label for CAT, indicated that the enzyme from pigeon breast muscle has a single acylcarnitine-binding site per protein molecule (Chase & Tubbs, 1970). Since the details of the molecular event(s) taking place within the binding site in the photoinhibition of CAT by PAP-TC are largely unknown, prediction of the exact stoichiometry of covalent incorporation of the analogue was not straightforward. With 30 mM-2-mercaptoethanol present, photolysis of solutions of CAT and PAP-[14 C]TC resulted in an extrapolated estimate of 1.2 ± 0.1 mol of photolabel bound/mol of CAT inactivated (Fig. 3). Covalently incorporated PAP-TC is thus quite effective in blocking the CAT active site at nearly the theoretical 1:1 enzyme/analogue stoichiometry.

Irreversible photoincorporation of PAP-[14 C]TC into CAT

In a separate demonstration that irreversible binding of PAP-TC to CAT occurs upon photolysis, enzyme that had been photolysed in the presence of 7 μ M-PAP-[14 C]TC was subjected to SDS/polyacrylamide-gel electrophoresis followed by autofluorography (Mauro, 1983). An

Fig. 3. Determination of the stoichiometry of photoincorporation of PAP-TC into CAT

The error bars represent the S.E.M. for triplicate measurements. The concentration of enzyme in photolysed solutions was 350 μ g/ml and the concentrations of PAP-TC tested were 5, 10, 15, 20 and 30 μ M; other details are given in the Experimental section.

Table 1. Inhibition of CAT with photoactivated PAP-TC in the presence of 30 mM-2-mercaptoethanol

See the Experimental section for details.

PAP-TC(μ M)	Acetyl-DL-carnitine (mM)	Inhibition (%)
10	0	12
20	0	28
40	0	40
10	5.0	2
20	5.0	6
40	5.0	8

autofluorogram of the stained and dried gel showed a single radioactive band that coincided with the single protein band that was detected by Coomassie Brilliant Blue stain. The band had a calculated M_r of 55000, identical with the published value for CAT (Chase & Tubbs, 1969). These results strongly indicated that PAP- ^{14}C TC had been covalently incorporated into the enzyme upon photolysis. This experiment also demonstrated that the thioether link between the protein-bound aryl group and the ^{14}C -containing thiocarnitine group was stable under the conditions used to conduct the electrophoretic separation and to subsequent gel-processing steps. In addition, the electrophoresis results showed that the CAT obtained from Sigma Chemical Co. was free of any significant protein contaminants.

Peptide maps for photolabelled CAT

As a further means of demonstrating that the photoreagent was incorporated at the active site, peptide maps were prepared for CAT that had been photolabelled with PAP- ^{14}C TC in the presence and in the absence of acetyl-DL-carnitine (Fig. 4). Amino acid analysis of CAT revealed that the enzyme contains 4.9 mol% arginine and 7.7 mol% lysine residues (Table 2). CAT is a monomer (M_r 55000) containing approximately 500 amino acid residues. By using the above values for the content of arginine and lysine, we calculated about 25 arginine and 38 lysine residues per molecule of intact CAT. Thus it can be estimated that about 65 peptide fragments should be generated upon exhaustive trypsin treatment of CAT.

The h.p.l.c. peptide map (Fig. 4) indicates that several peptides (about six) obtained from exhaustive trypsin digestion of photolabelled CAT contain bound PAP-

Table 2. Amino acid analysis of acid-hydrolysed CAT

See the Experimental section for details. Results are given as means $\pm$ s.e.m. for three trials. Abbreviation: N.D., not determined (tryptophan and proline are destroyed during acid hydrolysis).

Amino acid	Composition (mol%)
Asx	11.5 $\pm$ 0.6
Cys	0.50 $\pm$ 0.0
Glx	11.0 $\pm$ 0.8
Ser	19.5 $\pm$ 1.3
His	2.4 $\pm$ 0.5
Gly	3.0 $\pm$ 0.3
Thr	3.2 $\pm$ 0.6
Arg	4.9 $\pm$ 0.7
Ala	10.2 $\pm$ 0.4
Tyr	3.3 $\pm$ 0.1
Met	2.3 $\pm$ 1.3
Val	3.4 $\pm$ 0.8
Phe	4.1 $\pm$ 0.6
Ile	1.3 $\pm$ 0.4
Leu	10.9 $\pm$ 2.4
Lys	7.7 $\pm$ 0.5
Trp	N.D.
Pro	N.D.

^{14}C TC in amounts significantly above background. It is noteworthy that very little incorporation of the radioactive photoprobe was observed in the group of largely unresolved peptides that emerged early in the propan-1-ol

Fig. 4. Separation of tryptic peptides from CAT inhibited by photolysis with PAP- ^{14}C TC

The tryptic digest was injected on an Alltech C_{18} column (4.6 mm $\times$ 250 mm) in 0.25 M-acetic acid/0.1 M-pyridine buffer, pH 4.0, and eluted with a propan-1-ol gradient. The gradient was 0–19% (v/v) propan-1-ol in 120 min and 19–40% (v/v) propan-1-ol in 60 min. The continuous trace is the fluorescence detection; the histogram represents ^{14}C radioactivity.

gradient, i.e. the more hydrophilic peptides. Almost all of the radioactivity was associated with the more hydrophobic peptides, which were eluted later in the propan-1-ol gradient.

An experiment identical with the above, with the exception that 5 mM-acetyl-DL-carnitine was present as a site protector in the photolysed solution of CAT and PAP-TC, was performed. Under these conditions, total incorporation of PAP-[¹⁴C]TC above background was decreased to approx. 15% of its former value. The small amount of analogue covalently bound under these conditions appeared at the same points in the elution profile as shown in Fig. 4, suggesting that all labelled peptides are photolabelled as a consequence of the reagent being bound at the active site (Mauro, 1983).

The labelling of a number of peptides within a binding site by a photoaffinity label is not uncommon (Fleet *et al.*, 1969; Guillory, 1979). The so-called 'shotgun effect' as described by Singer (1967) asserts that a nitrene may insert into several places in a binding site depending upon photoactivation. The tryptic map of a photolabelled enzyme may therefore yield several labelled peptides. It should be noted, however, that photolabelling of a protein kinase with 8-azido cyclic AMP led to only one site of photoincorporation (Kerlavage & Taylor, 1980). The use of racemic PAP-[¹⁴C]TC in the photolabelling experiments may have increased the number of labelled peptides. Chase & Tubbs (1966) have observed that the binding constant of acetyl-D-carnitine is virtually identical with that of acetyl-L-carnitine, although only the latter is catalytically competent. It is likely that both stereoisomers of PAP-TC bind to CAT. As a consequence, the *p*-azidophenacyl group should bind in the active site in two different orientations with respect to the thiocarnitine moiety. This postulate is supported by the observation by Chase & Tubbs (1970) that bromoacetyl-L-carnitine is an irreversible inhibitor of CAT but that bromoacetyl-D-carnitine is not. Clearly, repeating the photolabelling experiments with optically resolved PAP-TC enantiomers would be informative. However, to date we have been unable to resolve DL-thiocarnitine completely (Dühr *et al.*, 1983). Finally, it is conceivable that the presence of the incorporated agent interferes with tryptic cleavage at one or two sites, lending to incomplete cleavage. Such an effect would also increase the number of detected labelled peptides.

Concluding comments

Chase (1967) reported that CAT from pigeon breast muscle demonstrates nearly identical Michaelis constants for the binding of L-carnitine esters with acyl groups of chain length C₂–C₁₀ (*V*_{max} varies dramatically, however). On the basis of the size of the azide-substituted ring, we estimated that the *p*-azidophenacyl group would be roughly equivalent in length to a C₆ alkyl chain. It therefore seemed likely that PAP-TC would readily fit within the acylcarnitine-binding site on the enzyme. Indeed, this was observed. Lacking detailed structural information about the CAT molecule (i.e. X-ray-crystallographic analysis), the exact nature of the stereoelectronic interactions of the photoprobe within the CAT binding site are unknown, so that the anomalously low-binding constant observed for PAP-TC in competition with L-carnitine must be considered an unexplained phenomenon at this time.

Our results indicate that PAP-TC is covalently

photoincorporated into the carnitine/acylcarnitine-binding site on CAT. The stoichiometry of photoincorporation is 1 mol of reagent/mol of enzyme, i.e. the expected value. Tryptic peptide maps indicate that several peptides are photolabelled, however; photoincorporation in each labelled peptide is diminished by the presence of the substrate protector, acetyl-DL-carnitine. Thus the binding specificity of the reagent appears to be quite good, but the specificity of photoincorporation appears to be poor in that it involves a number of residues.

Besides CAT, several other interesting biological systems are known to bind carnitine and/or carnitine esters. These include several enzymes (cf. Frenkel & McGarry, 1980), the carnitine/acylcarnitine translocase (Noel *et al.*, 1985) and a cardiac carnitine-binding protein (Cantrell & Borum, 1982). We anticipate that PAP-TC can be a useful tool for investigating these systems.

We are grateful to Purnima Ray and Edward Dühr for technical assistance and to Dr. Boyd Haley for consultations and for critically reading this manuscript. This work was supported by research grants from the National Institutes of Health (GM 20077 to R.E.B.; NS 17647 to R.V.L.). R.V.L. is an Alfred P. Sloan Fellow (1983–1985) and the recipient of a Research Career Development Award (NS 00968).

REFERENCES

- Bayley, H. & Knowles, J. R. (1977) *Methods Enzymol.* **46**, 69–114
- Bayley, H. & Knowles, J. R. (1978) *Biochemistry* **17**, 2414–2419
- Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 1762–1771, Verlag Chemie, Weinheim
- Bohlen, P., Stein, S., Stone, J. & Udenfriend, S. (1975) *Anal. Biochem.* **67**, 438–445
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Cantrell, C. R. & Borum, P. R. (1982) *J. Biol. Chem.* **257**, 10599–10604
- Chase, J. F. A. (1965) *Methods Enzymol.* **13**, 387–393
- Chase, J. F. A. (1967) *Biochem. J.* **104**, 510–518
- Chase, J. F. A. & Tubbs, P. K. (1966) *Biochem. J.* **99**, 32–40
- Chase, J. F. A. & Tubbs, P. K. (1969) *Biochem. J.* **111**, 225–235
- Chase, J. F. A. & Tubbs, P. K. (1970) *Biochem. J.* **116**, 713–720
- Chase, J. F. A., Pearson, D. J. & Tubbs, P. K. (1965) *Biochim. Biophys. Acta* **96**, 162–165
- Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol.* **29**, 1–23
- Crestfield, A. M., Moore, S. & Stein, W. H. (1963) *J. Biol. Chem.* **238**, 622–627
- Dühr, E. F., Mauro, J. M., Clennan, E. L. & Barden, R. E. (1983) *Lipids* **18**, 382–386
- Fleet, G. W. J., Porter, R. R. & Knowles, J. R. (1969) *Nature (London)* **224**, 511–512
- Frenkel, R. A. & McGarry, J. D. (eds.) (1980) *Carnitine Biosynthesis, Metabolism, and Functions*, Academic Press, New York
- Guillory, R. J. (1979) *Curr. Top. Bioenerg.* **9**, 267–414
- Hixson, S. S. & Hixson, S. H. (1973) *Photochem. Photobiol.* **18**, 135–138
- Hixson, S. H. & Hixson, S. S. (1975) *Biochemistry* **14**, 4251–4254
- Jones, B. N., Paabo, S. & Stein, S. (1981) *J. Liq. Chromatogr.* **4**, 565–586
- Kerlavage, A. R. & Taylor, S. S. (1980) *J. Biol. Chem.* **255**, 8483–8488
- Koide, A., Koiti, T., Ericsson, L. J., Kumar, S., Neurath, H. & Walsh, K. S. (1978) *Biochemistry* **17**, 5657–5672
- Lau, E. P., Haley, B. E. & Barden, R. E. (1977) *Biochemistry* **16**, 2581–2585

- Lewis, R. V. (1979) *Anal. Biochem.* **98**, 142–145
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Maassen, J. A. & Moller, W. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1277–1280
- Mauro, J. M., (1983) Ph.D. Thesis, University of Wyoming
- Mauro, J. M., Duhr, E. F. & Barden, R. E. (1983) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42**, 1785
- Nishimura, J. S., Mitchell, T., Hill, K. A. & Collier, G. E. (1982) *J. Biol. Chem.* **257**, 14896–14902
- Noel, H., Goswami, T. & Pande, S. V. (1985) *Biochemistry* **24**, 4504–4509
- Owens, J. R. & Haley, B. E. (1976) *J. Supramol. Struct.* **5**, 91–102
- Ruoho, A. E., Kiefer, H., Roeder, P. E. & Singer, S. J. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2567–2571
- Sigma-Tau Industrie Farmaceutiche Riunite (1982) Belg. Patent 891615; cited in *Chem. Abstr.* (1982) **97**, 182859
- Simon, E. J. & Shemin, D. (1953) *J. Am. Chem. Soc.* **75**, 2520
- Singer, S. J. (1967) *Adv. Protein Chem.* **22**, 1–54
- Venkatraghavan, V. & Smith, O. J. (1983) *Arch. Biochem. Biophys.* **220**, 193–199
- Ziegler, H. J., Bruckner, P. & Binon, F. (1967) *J. Org. Chem.* **32**, 3989–3991

Received 16 December 1985/10 February 1986; accepted 25 March 1986