Identification of Anesthetic Binding Sites on Human Serum Albumin Using a Novel Etomidate Photolabel*

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We have synthesized a novel analog of the general anesthetic etomidate in which the ethoxy group has been replaced by an azide group, and which can be used as a photolabel to identify etomidate binding sites. This acyl azide analog is a potent general anesthetic in both rats and tadpoles and, as with etomidate, is stereoselective in its actions, with the R(+)-enantiomer being significantly more potent than the S(−)-enantiomer. Its effects on α1β2γ2s GABAA receptors expressed in HEK-293 cells are virtually indistinguishable from the parent compound etomidate, showing stereoselective potentiation of GABA-induced currents, as well as direct mimetic effects at higher concentrations. In addition, a point mutation (β2 N265M), which is known to attenuate the potentiating actions of etomidate, also blocks the effects of the acyl azide analog. We have investigated the utility of the analog to identify etomidate binding sites by using it to photolabel human serum albumin, a protein that binds ~75% of etomidate in human plasma and which is thought to play a major role in its pharmacokinetics. Using HPLC/mass spectrometry we have identified two anesthetic binding sites on HSA. One site is the well-characterized drug binding site I, located in HSA subdomain IIA, and the second site is also an established drug binding site located in subdomain IIB, which also binds propofol. The acyl azide etomidate may prove to be a useful new photolabel to identify anesthetic binding sites on the GABA receptor or other putative targets.

Although it is now widely accepted that general anesthetics exert their effects by binding directly to their protein targets (1–3), information on the precise molecular locations of these binding sites has been slow in coming. Most information has been derived from in vitro electrophysiological experiments in which putative anesthetic targets, ion channels, or receptors, are genetically modified. What this approach provides is information on the molecular determinants of anesthetic sensitivity, but it is usually impossible to tell whether these determinants represent portions of anesthetic binding sites or regions of the channel or receptor that are responsible for transducing anesthetic binding into changes in channel gating. In principle, the most direct approach would be to determine a high resolution crystal structure of the ion channel or receptor in question in the presence and absence of anesthetics. Unfortunately, because of the difficulties in crystallizing membrane proteins, such experiments are still some way off. An alternative strategy that has recently had some success is to use anesthetics that have been modified so that they contain photolabile groups. When these anesthetic analogs are illuminated with a bright light, they are converted into highly reactive intermediates, which then, hopefully, bind irreversibly to their targets.

The idea of using photoaffinity labeling to identify anesthetic binding sites was first implemented using the volatile anesthetic halothane (4, 5). Halothane breaks down to form highly reactive ethane radicals when illuminated with ultraviolet light at wavelengths around 250 nm, but at these wavelengths there is likely to be some damage to the protein targets themselves. Subsequent work has attempted to improve on these pioneering studies by synthesizing anesthetic analogs with incorporated diazirine groups (6, 7). The diazirine moiety absorbs light at longer wavelengths and has been widely used in photolabeling studies (8) and a number of different photoactivatable anesthetics have been synthesized. A diazirine alcohol derivative, 3-(2-hydroxyethyl)-3-n-pentyl diazirin (azioctanol) has been synthesized and characterized (7) and shown to label the nicotinic acetylcholine receptor from Torpedo (9). Two regioisomers of octanol bearing a diazirine group on the third and seventh carbon (3- and 7-azioctanol, respectively) have been used to locate and delineate an anesthetic binding site on adenylate kinase (10) and protein kinase Cδ (11). A diazirine derivative of the intravenous anesthetic etomidate has also been synthesized (12). This photolabel has been shown to bind to the nicotinic acetylcholine receptor from Torpedo (12, 13) as well as to the GABA receptor (14). In the latter case, two particular methi-

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EXPERIMENTAL PROCEDURES

Synthesis of Acyl Azide Etomidate—Fig. 1 shows the molecular structure of the acyl azide analogs of etomidate and the parent compound etomidate, together with the reaction scheme for their synthesis. Both isomers (R(+) and S(-)) of this compound were produced using the same synthetic route (see Fig. 1B) but with starting material (methyl benzylamine) of the appropriate chirality in each case. Methyl benzylamine (I) was treated with triethyl amine and ethyl chloroacetate in dimethylformamide. The resulting methylbenzyl glycin ethyl ester (II) was distilled to furnish pure product as a yellow oil (53% yield, b.p. 154–160 °C/0.63 mm Hg). This was then treated with freshly prepared sodium methoxide and methyl formate in tetrahydrofuran at 10 °C, followed by reaction of the resulting c-formyl derivative with hydrochloric acid-potassium thiocyanate resulting in the formation of the thiol (III).

Oxidative desulfurization of the thiol (III) in the presence of nitric acid-sodium nitrite afforded the desired methyl ester (IV) in 74% yield as a viscous orange oil, which was used without further purification. This was refluxed for 24 h with four equivalents of hydrazine hydrate in ethanol to yield the hydrazide (V) as a pale yellow oil (83% yield). Subsequent reaction of this hydrazide with nitrous acid resulted in the desired product acyl azide (VI) in moderate yield (40%) as an off-white solid (m.p. 58 °C). If too much hydrochloric acid was used in this last step then treated with triethyl amine, isolated. The free acyl azide could be obtained from this by treatment with di-isopropylamine.

UV absorption spectra of compounds were obtained using a Beckman DU650 spectrophotometer. Octanol-water partition coefficients were measured by weighing compounds directly into disposable UV-transparent cuvettes (Brand, Wer-
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| Compound | Structure | Melting point (°C) | $K_{OW}$ | Log $K_{OW}$ |
|----------|-----------|-------------------|----------|-------------|
| Etomidate | | 62 | 776 ± 9 | 2.89 |
| Acyl azide | | 46 | 513 ± 5 | 2.71 |
| Methoxy ester | Oil at room temp. | | 191 ± 7 | 2.28 |
| Alcohol | | 101 | 19 ± 1 | 1.28 |
| Aldehyde | | 79 | 59 ± 5 | 1.77 |

**Solutions**—$R(\pm)$-etomidate was obtained as Hypnomidate (2 mg ml$^{-1}$ in 35% propylene glycol; Janssen-Cilag Ltd, High Wycombe, UK). $S(\pm)$-etomidate was synthesized according to previously described procedures (20) with minor modifications. For all experiments, $S(\pm)$-etomidate solutions were made up using a stock solution of 2 mg ml$^{-1}$ in 35% propylene glycol. For the electrophysiological and photolabeling experiments, acyl azide solutions were made up using concentrated stocks in ethanol. The highest concentration of ethanol used (34 mM) was found to have no effect on GABA-activated currents. In the photolabeling experiments, an equivalent amount of ethanol was added to the control solutions. For the in vivo potency measurements, solutions containing the acyl azide were made up from 2 mg ml$^{-1}$ stock solutions in 35% propylene glycol (tadpole experiments) or from stocks in 100% propylene glycol (up to 6 mg ml$^{-1}$; rat experiments). In both sets of in vivo experiments, the carrier was found to have no effect by itself.

Solutions containing GABA were prepared using concentrated stocks made up in saline on the day of the experiment. All chemicals were obtained from Sigma unless otherwise stated.

**Cell Culture**—Modified HEK-293 cells (tsA 201) were maintained in 5% CO$_2$, 95% air in a humidified incubator at 37 °C in growth media (89% Dulbecco's modified Eagle's medium; 10% heat-inactivated fetal bovine serum; 1% penicillin (10,000 units ml$^{-1}$) and streptomycin (10 mg ml$^{-1}$)). When the tsA cells were 80% confluent, they were split and plated for transfection onto glass coverslips coated with poly-d-lysine to ensure good cell adhesion.

The HEK-293 cells were transiently transfected with human cDNAs coding for $\alpha_1$, $\beta_2$, and $\gamma_2$s GABA$_A$ subunits using the calcium phosphate method. The coding sequences for these three subunits were subcloned into pcDNA3.1 (Invitrogen, Paisley, UK), a vector designed for high expression in mammalian cells. 1 μg of cDNA encoding each subunit was added to each 35-mm diameter well, and 1 μg of a plasmid encoding the cDNA of green fluorescent protein was included to identify cells expressing GABA$_A$ receptor cDNAs. Following a 24-h incubation period at 3% CO$_2$ the cells were rinsed with saline and fresh growth medium was added to the wells. The cells were incubated at 37 °C with 5% CO$_2$/95% air for 12–72 h before electrophysiological measurements were made.

**Electrophysiology**—Ionic currents evoked by the application of GABA were recorded using the whole cell patch-clamp technique with an Axopatch 200A amplifier (Axon Instruments, Union City, CA). Recording pipettes were pulled from thin-walled borosilicate glass capillaries (Harvard Apparatus, Edenbridge, Kent, UK) using a two-stage vertical puller (Narishige, Tokyo, Japan). After brief fire-polishing, pipettes were back-filled with 0.2 μm-filtered intracellular solution (140 mM CsCl, 1 mM MgCl$_2$, 11 mM EGTA, 10 mM HEPES, titrated to pH 7.2 with CsOH). These pipettes, with typical resistances of 2.5–4 MΩ, readily formed “giga-ohm” seals with the cells, which upon

**TABLE 1**

Octanol-water partition coefficients and melting points for etomidate and related analogs
establishing the whole cell configuration were voltage-clamped at −60 mV. Series resistance was compensated by 80–90%. During recording, cells were bathed in an extracellular solution containing 145 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 1.5 mM CaCl₂, 5.5 mM D-glucose, 10 mM HEPES, (titrated to pH 7.4 with NaOH). All drugs were applied locally to the cell via a double-barreled glass capillary tube as described previously (21). Recordings were filtered at 50 Hz (−3 dB) using an 8-pole Bessel filter (Frequency Devices, Lyons Instruments, Hertfordshire, UK) and digitized at 200 Hz before being stored on a computer hard disk. Data were subsequently analyzed offline using Clampfit software (Axon Instruments, Union City, CA). All electrophysiological measurements were carried out at room temperature (22 ± 1 °C).

Agonist-containing test solutions were typically applied to the cell for 2–5 s. GABA concentration-response data were then fitted using a least squares method to the Hill equation shown in Equation 1.

\[
I = \frac{I_{\max} \cdot [\text{GABA}]^n}{[\text{GABA}]^n + EC_{50}^n}
\]  
(Eq. 1)

For experiments involving anesthetics, test solutions contained the appropriate concentrations of GABA and the anesthetic. High concentrations of anesthetic were found to directly activate receptors in the absence of GABA and therefore to separate this GABA-mimetic effect from the GABA-modulatory effect, the anesthetic was applied before, as well as during, the application of GABA. Anesthetics were typically pre-applied for at least 30 s to ensure that a stable baseline current had been obtained, before application of the GABA-containing test solution. At low concentrations (3 μM or less for R(+)-etomidate), there was no GABA-mimetic effect and pre-application of anesthetics was found to be unnecessary. GABA concentration-response curves in the presence of anesthetic were constructed that were fitted with a Hill equation as before. Anesthetic potentiation (P) was defined by Equation 2 where \(I_0\) is the peak of the control GABA-induced current, and \(I\) is the peak of the GABA-induced current in the presence of anesthetic.

\[
P(\%) = 100 \times \left(1 - \frac{I}{I_0}\right)
\]  
(Eq. 2)

Values throughout this article are given as means ± S.E.

**In Vivo Anesthetic Potency Measurements**—All experiments were performed in compliance with the United Kingdom Animals (Scientific Procedures) Act of 1986.

**Potency Measurements in Tadpoles**—General anesthetic potencies of compounds were determined for 4–6-week-old Rana temporaria tadpoles (Blades Biological, Cowden, Kent, UK) in the pre-limb-bud stage of development. Tadpoles were maintained in an aerated aquarium at 20–22 °C. Groups of 8–12 tadpoles were placed in 400-ml glass beakers containing 300 ml of the anesthetic solution. Stock solutions of acyl azide etomidate were usually made up in the same carrier as used clinically i.e. 2 mg ml⁻¹ in 35% propylene glycol. However, for the highest concentrations of some compounds, a more concentrated stock, 3 mg ml⁻¹, was utilized. The highest concentration of propylene glycol used was 71 mM, which was found to have no effect by itself. The anesthetic end point was defined as the lack of a purposeful and sustained swimming response after a gentle inversion with a smooth glass rod. This was assessed at 10-min intervals over a period of 60 min by which time equilibrium had been reached. After the experiment, tadpoles were returned to fresh tap water where their recovery was monitored. In most cases, normal swimming activity was restored within 1 h. Tadpoles that did not recover were excluded from the analysis. Tadpole concentration-response data were fitted according to the method of Waud (22) with a logistic equation of the form in Equation 3,

\[
P = \left(\frac{100C^n}{C^n + (EC_{50})^n}\right)
\]  
(Eq. 3)
Anesthetic solutions were prepared in the same carrier as used and water and were maintained on a 12 h light-dark cycle. To ensure a minimal "dead volume" (PTFE tubing using a specially designed stainless steel fitting to Oxford, UK). The cannula was connected to a short length of veins using a 24-gauge, 19-mm cannula (Becton Dickinson, administered intravenously through one of the two lateral tail don, UK) weighing

Potency Measurements in Rats—Experiments were performed on male Sprague-Dawley rats (B & K Universal, London, UK) weighing ~300 g. Rats were given free access to food and water and were maintained on a 12 h light-dark cycle. Anesthetic solutions were prepared in the same carrier as used clinically, i.e. 35% propylene glycol in water. Anesthetics were administered intravenously through one of the two lateral tail veins using a 24-gauge, 19-mm cannula (Becton Dickinson, Oxford, UK). The cannula was connected to a short length of PTFE tubing using a specially designed stainless steel fitting to ensure a minimal "dead volume" (<15 µl). Solutions were delivered from a 2-ml gas tight syringe (Precision Sampling Corp., Baton Rouge, LA) mounted on a computer-controlled syringe pump. Most experiments were performed using a standard injection protocol that delivered 600 µl of solution over 24 s at a rate of 1500 µl min⁻¹. Animals were restrained during the injection in a specially made plexiglass tube. Immediately after the injection, the animal was placed carefully on its back and was judged as anesthetized if it failed to completely right itself (all four paws on the ground) within 1 min from the start of the injection. Any animal that succeeded in righting itself during this period was scored as awake. Animals were used only once

and experiments were carried out at an ambient temperature of 22 ± 1 °C. Quantal dose-response data were fitted to the same logistic equation as before using the Waud method (22).

Photolysis of Acyl Azide—For photolabeling experiments, solutions of HSA were made up in a 1-ml volume of phosphate-buffered saline (170 mM NaCl, 4 mM KCl, 10 mM Na₂HPO₄, titrated to pH 7.4 using HCl), before addition of the acyl azide etomidate as a 500 µM stock solution made up in ethanol. For control experiments in the absence of the acyl etomidate, the equivalent amount of ethanol was added instead. Samples were then preincubated for 5 min before transfer to a 4-ml quartz cuvette for exposure to UV light. The cuvette was placed in a polished stainless steel holder for exposures using a 500 watt mercury (xenon) lamp (Lot-Oriel, Leatherhead, Surrey, UK). The light produced by this source was transmitted via a dichroic mirror (280–400-nm reflection band) to remove excess IR radiation, before passing through an interference filter onto the cuvette. Most experiments were performed using a >320 nm band-pass filter (Lot-Oriel). Exposures were controlled using a manual shutter. Samples were continuously mixed during exposure using a small magnetic stirrer.

Trypsin Digestion—Following UV exposure, 0.5 ml of the sample was dried down to a volume of about 50 µl using a rotary evaporator (DNA Plus; Jouan Nordic, Denmark), before addi-
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**RESULTS**

Both enantiomers of acyl azide etomidate were synthesized to a high degree of purity (>99%) and the ultraviolet spectra showed a strong absorption band centered at 270 nm (Fig. 1C). (This is in contrast to etomidate which shows a strong absorption band at 240 nm.) The extinction coefficient at the maximum absorption was $\epsilon_{270} = 15,500 \text{ M}^{-1} \text{ cm}^{-1}$. The absorption at longer wavelengths was still substantial (e.g. $\epsilon_{320} = 120 \text{ M}^{-1} \text{ cm}^{-1}$) and illumination using a band-pass filter ($\lambda > 320 \text{ nm}$) caused rapid photolysis (Fig. 1C). The time course of photoconversion was roughly exponential with a half-time $\tau \approx 8 \text{ s}$. Acyl azide etomidate was stable in aqueous solution, with less than 10% hydrolysis after 4 h. The replacement of the ethyl ether on etomidate for the acyl azide group had very little effect on the overall polarity of the molecule, and the octanol/water partition coefficients were comparable. Table 1 gives the octanol/water partition coefficients for etomidate, acyl azide etomidate, and some related analogs for comparison.

Acyl azide etomidate also displayed a similar in vivo pharmacology to that of the parent compound. This is illustrated by the data in Fig. 2A, which show that the EC$_{50}$ concentration for loss of righting reflex in tadpoles is $11 \pm 2 \mu\text{M}$ for the $R(+)$ enantiomer and significantly greater for the $S(-)$ enantiomer ($36 \pm 7 \mu\text{M}$), as is the case for etomidate (23). The $R(+)$ acyl azide eto-
midate also caused rapid and reversible loss of righting reflex in rats (Fig. 2B) with an ED_{50} dose of 1.7 ± 0.2 mg kg^{-1}.

The in vitro pharmacology of acyl azide etomidate was virtually indistinguishable from that of etomidate. This is illustrated by the data in Fig. 3 for α1β2γ2s GABA_A receptors expressed in HEK-293 cells. Both R(+) etomidate and R(+) acyl azide etomidate caused a marked leftwards shift in GABA concentration-response curves. A Hill equation fitted to the control data gave an EC_{50} of 56 ± 3 μM and a Hill coefficient of n_H = 1.3 ± 0.1. These parameters were 11 ± 4 μM and 0.6 ± 0.1 in the presence of 3 μM R(+) etomidate and 11 ± 5 μM and 0.6 ± 0.2 in the presence of 3 μM R(+) acyl azide etomidate. The S(-) enantiomers had no significant effect on the GABA concentration-response curve (Fig. 3, A and B). Both R(+) etomidate and R(+) acyl azide etomidate caused a concentration-dependent and saturable potentiation of GABA-evoked currents (Fig. 3, C and D), which was abolished in receptors which carried the N265M mutation in the β2 subunit (insets to Fig. 3, C and D). This mutation has been shown to block the potentiating action of etomidate (24). Finally, both R(+) etomidate and R(+) acyl azide etomidate caused a concentration-dependent activation of the α1β2γ2s GABA_A receptor in the absence of GABA. This GABA-mimetic effect showed no signs of saturating at high concentrations (Fig. 3, E and F).

Having established that R(+) etomidate and R(+) acyl azide etomidate shared very similar in vivo and in vitro pharmacological profiles, we went on to test the ability of R(+) acyl azide etomidate to photolabel a protein target, human serum albumin. This plasma protein is known to play an important role in the pharmacokinetics of etomidate, and ~75% of etomidate binds to plasma proteins following intravenous injection (19). We first determined the extent of binding of R(+) etomidate and R(+) acyl azide etomidate to HSA and measured dissociation constants of 176 ± 6 μM and 175% of etomidate binds to plasma proteins following intravenous injection (19). We first determined the extent of binding of R(+) etomidate and R(+) acyl azide etomidate to HSA and measured dissociation constants of 176 ± 6 μM and 175 ± 6 μM, respectively. A and B, these two panels show the UV absorbance chromatograms at 214 nm for the new peaks obtained at 40.3 min (A) and at 55.7 min (C). In both cases, the trace for the control experiment (HSA exposed to UV in the absence of the acyl azide) is the solid line, and the trace for the labeling experiment (HSA exposed to UV in the presence of the acyl azide) is the dotted line. The new peaks are indicated by the arrows. (C also shows that a new feature was observed at a retention time of 56.7 min. However, this peak was also present in a control experiment where the acyl azide was exposed to UV in the absence of HSA, showing that it is caused by the presence of the photolabel and is not a labeled peptide.) B and D, fractions corresponding to the two labeled peptides were subjected to tandem mass spectrometric analysis using a quadrupole time-of-flight (Q-TOF) instrument. For both labeled peptides, the doubly charged species were the predominant ions so these were selected for fragmentation and further analysis in the second stage of the mass spectrometer. Shown here are the fragment ion mass spectra for the labeled peptides obtained at 40.3 min (m/z = 580.5, B) and at 55.7 min (m/z = 671.4, D). Ion currents have been normalized to the most abundant species. In both cases, most of the major peaks in the spectrum can be assigned to the two most significant series of ions associated with fragmentation of the peptide backbone, the b and y series. From these series of ions, the sequences for the two labeled peptides can be identified for LKCASLQK for the peak obtained at 40.3 min and KQTALVELVK for the peak obtained at 55.7 min. The species labeled with an asterisk correspond to the protonated form of the fragment of the acyl azide (molecular weight 214) that was attached to these peptides at Lys^{199} and Lys^{523}.

![Identification of peptides photolabeled by acyl azide etomidate](image-url)
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The two labeled amino acids are found in two known HSA drug binding sites (see Fig. 7A). The first site is located in subdomain IIA, and the second site is located in subdomain IIIB. The apparent affinities for the peptides at 40.3 min and 55.7 min were 89 ± 2 μM and 106 ± 6 μM, respectively. Labeling of these two peptides by 25 μM acyl azide etomidate was reduced by 66 and 89%, respectively in the presence of a high concentration (1000 μM) of etomidate, confirming the label binds to the same sites as the parent compound.

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of the form $y = A^n/(A^n + (K_y^n))$ where $y$ is the normalized labeling, $A$ is the concentration of acyl azide, $n$ is the slope parameter, and $K_y$ is an apparent binding affinity (solid line for the peptide at 40.3 min and dashed line for the peptide at 55.7 min). In all cases, the data were normalized such that the time point corresponding to maximum abundance was unity.

121 ± 5 μM, respectively. We next developed a protocol using HPLC/MS that would allow the separation and identification of the peptides of HSA following tryptic cleavage (see “Experimental Procedures”). Of the 79 predicted peptides, we identified all but five, four of which were single amino acids. A typical separation is illustrated in Fig. 4.

For the photolabeling experiments, we first equilibrated HSA (150 μM) with R(+) acyl azide etomidate (1000 μM) in phosphate-buffered saline and then exposed the solution, while constantly stirring, to UV light with wavelengths >320 nm. (From our binding data, under these conditions, we can calculate that there would be 0.88 mol of acyl azide etomidate bound per mol of HSA prior to labeling.) Two photolabeled peptides were readily identified, one with a retention time of 40.3 min and one with a retention time of 55.7 min (see Fig. 5, A and C). The molecular weights of these labeled peptides were 1159.6 and 1340.9, respectively. Although the identity of the two labeled peptides could be deduced on the basis of their masses alone, we confirmed their identities using tandem mass spectrometry as 198-LKCASLQK-205 and 525-KQTALVELVK-534 (Fig. 5, B and D). Neither of these peptides would be expected on the basis of normal tryptic cleavage which indicated that the labeling was interfering with the tryptic digestion. The appearance of the two new peptides was accompanied by a reduction in the ion counts associated with only two peptides, CASLQK and QTALVELVK, by 17 and 40% respectively. (These reductions mean binding stoichiometries of 0.17 and 0.40 mol label per mol of HSA, which together with the average occupancy of 0.88 prior to labeling, allows us to calculate an average efficiency of labeling of 32%.) This implied the labeling was occurring at Lys525 and either Leu198 and/or Lys199. An analysis of the fragmentation pattern identified the two labeled amino acids as Lys199 and Lys525. The rate of labeling of the two peptides is illustrated in Fig. 6A, which shows the time course of labeling at two different concentrations of acyl azide etomidate (50 and 400 μM). For comparison, the abundance of the peptide containing the single tryptophan residue (Trp214) can be seen to remain essentially unchanged (Fig. 6B) during the course of the labeling, indicating little or no photodamage to the protein itself. The concentration dependence of labeling was determined by measuring the fraction of peptide labeled after 40 s UV exposure at varying concentrations of acyl azide etomidate. The results of these experiments are shown in Fig. 6C. The apparent affinities for the peptides at 40.3 min and 55.7 min were 89 ± 2 μM and 106 ± 6 μM, respectively. Labeling of these two peptides by 25 μM acyl azide etomidate was reduced by 66 and 89%, respectively in the presence of a high concentration (1000 μM) of etomidate, confirming the label binds to the same sites as the parent compound.

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Atomic coordinates of acyl azide etomidate were generated using a minimal energy algorithm (25), and the molecule was built into the HSA structure at the two labeled sites. The site in subdomain IIA also binds phenylbutazone, and this structure (2bxc) was used as a guide to position the acyl azide molecule. Similarly, the binding site in subdomain IIIB also binds thyroxine, and this structure (1hk1) provided a suitable template for the positioning of the acyl azide molecule at this location. For both acyl azide molecules, the nitrogen which converts to a nitrene upon illumination lies 2 Å from the labeled amino acids, Lys^{199} and Lys^{525}. Close up views of the two binding sites are illustrated in Fig. 7, B and C.

DISCUSSION

The synthesis of acyl azide etomidate is straightforward and yields a highly pure product with good aqueous stability. The replacement of the ethoxy group of etomidate with an azide group caused the major UV absorption band of etomidate to shift from 240 to 270 nm with a peak extinction coefficient of 15,500 M⁻¹ cm⁻¹. Longer wavelengths (>300 nm) are more appropriate for photolabeling proteins because of the damage that can be caused due to UV absorption by, predominantly, typtophan and tyrosine residues. However, the very high peak UV absorption of acyl azide etomidate means that there is still substantial absorption at wavelengths, which are unlikely to cause photodamage, and the extinction coefficient at 320 nm (120 M⁻¹ cm⁻¹) is comparable to those observed with more conventional photolabeling groups such as diazirines. Exposure of acyl azide etomidate to light at wavelengths >320 nm caused photoconversion to a nitrene, which was rapid (τ_{1/2} 8 s) and complete (Fig. 1).

The acyl azide analog was a potent general anesthetic in both tadpoles and rats and displayed a similar potency and degree of stereoselectivity (Fig. 2) to that seen with the parent compound etomidate (23), as might be predicted from its similar octanol/water partition coefficient (Table 1). It was also essentially indistinguishable to etomidate in its actions on GABA₁ receptors (Fig. 3). One can be reasonably confident, therefore, that the binding of acyl azide etomidate to target proteins will accurately reflect the binding of etomidate itself.

We explored this using human serum albumin, a plasma protein that is known to bind etomidate to such an extent that it is likely to play a significant role in the pharmacokinetics of the drug (19). Following illumination of HSA in the presence of acyl azide etomidate and tryptic cleavage, two peptides were found to be modified, and fragmentation analysis identified the labeled amino acids as Lys^{199} and Lys^{525}. In both cases, the labeling blocked expected trypsin cleavages, which would normally have occurred after these lysines. The two peptides followed an almost identical time course of labeling (see Fig. 6A), which was essentially complete after 40 s. Over this time there was no significant change (see Fig. 6B) in the abundance of the peptide, which contained the single tryptophan in HSA (Trp^{214}), an indication that there was no significant photodamage to the protein itself at the wavelengths used for the labeling. Labeling at different acyl azide concentrations gave apparent affinities for the binding sites of 89 ± 2 μM and 106 ± 6 μM. These numbers, however, reflect both the binding affinity as well as the rate of the irreversible binding reaction so cannot be interpreted as true binding constants.

The two labeled amino acids lie within known drug binding sites on HSA. The first site in subdomain IIA is known as drug binding site I in the HSA literature (18) and has been shown to bind a diverse range of drugs. The second binding site, found in subdomain IIIB is also an established binding site (18), which is more selective than drug binding site I, and which has been shown to accommodate thyroxine (26) and the general anesthetic propofol (27).

In summary, we have shown how a novel etomidate photolabel can be used to identify etomidate binding sites on human serum albumin. Acyl azide etomidate may have future utility as a selective label for etomidate binding sites on the GABA₁ receptor, the likely target for etomidate in the central nervous system (28). Very recently (14), a diazirine derivative of etomidate has been shown to react with two...

![Identification of Etomidate Binding Sites on HSA](image-url)
methionine residues (αM236 and βM286) on the GABA<sub>A</sub> receptor, and our novel photolabel provides an alternative reagent to investigate etomidate binding sites on this receptor and other putative targets.

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