Kinetics of inhibition of purified and mitochondrial cytochrome c oxidase by psychosine (β-galactosylsphingosine)

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1. Psychosine (β-galactosylsphingosine) is the toxic agent in Krabbe's disease (globoid cells leukodystrophy). It inhibits purified bovine heart mitochondrial cytochrome c oxidase; there is a rapid phase of inhibition (complete within 10–15 s) and a slower phase (complete within 10–15 min). Both phases are also seen in rat liver mitochondria. IC₅₀ is about 200 µM psychosine in the purified enzyme and less than 20 µM in mitochondria. Psychosine inhibition is due to binding to cytochrome oxidase, not cytochrome c. 2. Bovine heart submitochondrial particles show inhibition similar to rat liver mitochondria. However, although proteoliposomes containing bovine heart cytochrome oxidase show an identical fast phase, they have no noticeable slow phase of inhibition. Addition of phospholipid liposomes to submitochondrial particles relieved the majority of psychosine inhibition, consistent with the removal of those molecules binding in the slow phase. Psychosine can inhibit cytochrome oxidase molecules facing in either direction in proteoliposomes and submitochondrial particles, suggesting that it can rapidly interact with both sides of a membrane when added externally. 3. At high ionic strength, the presence of psychosine decreases the Vₘₐₓ of cytochrome oxidase with little effect on the Kₘ for cytochrome c. This non-competitive inhibition suggests that the psychosine–enzyme complex is kinetically inactive and not labile over the time course of the assay. Psychosine does not inhibit the reduction of haem a or haem a₃ by artificial electron donors, but does inhibit the reduction of haem a by cytochrome c.

INTRODUCTION

Recently, attention has been drawn to the fact that damage to mitochondrial enzymes may impair mammalian brain function. There are some conditions where the primary site of damage appears to be at the level of mitochondrial cytochrome c oxidase. Several central nervous system disorders are caused by deficiency in cytochrome oxidase, e.g. Menkes' disease, Leigh's syndrome and Alper's disease [1]. Brain cytochrome oxidase may be damaged in ischaemia, either directly or via lipid peroxidation of its essential tightly bound cardioliopin molecules [2]. Furthermore, there is a non-specific hydrophobic binding site on the enzyme that may be close to the substrate (cytochrome c)-binding site [3]. This may be a specific target for inhibition by free radicals produced by iron–lipid complexes and thus explain the toxicity of some antitumour drugs, e.g. adriamycin [4]. Similar strong interactions occur with general anaesthetics such as lidocaine [5] and non-esterified fatty acids such as palmitate [6]. It is suggested that methanol toxicity is caused by the production of excess formate, a potent inhibitor of cytochrome oxidase [7].

The brain lipid, psychosine (β-galactosylsphingosine), which accumulates in a lipid-storage disorder (Krabbe's disease) is likely to be the cytotoxic agent in this neurological disorder [8], leading to a rapid and almost total disappearance of oligodendroglial cells. Psychosine is known to be both haemolytic [9] and to inhibit kinase C [10] and CTP:phosphocholine cytidyltransferase [11]. It also inhibits respiration in brain slices [9] and cytochrome c oxidation in rat liver mitochondria [12]. We have confirmed that this latter inhibition is due to a direct interaction between psychosine and cytochrome oxidase, generating an inactive complex that cannot oxidize cytochrome c effectively. This binding site may be similar to that for other hydrophobic inhibitors of the enzyme, and thus provides information as to the complex interactions of this membrane protein with its lipid milieu [3], as well as having implications for the study of Krabbe's disease.

EXPERIMENTAL

Cytochrome c oxidase was purified from beef hearts as described previously [13] with Tween-80 substituting for Emasol as the final detergent. Keilin–Hartree submitochondrial particles were prepared from minced hearts essentially as described [14] with the modifications described in [15]. Rat liver mitochondria were prepared by standard procedures [16]. Liposomes and cytochrome c-loaded cytochrome oxidase proteoliposomes were prepared [17] using 'asolectin' type (Sigma Type IV-S, 'phosphatidylcholine'). Psychosine was stored as a stock solution (10–20 mg/ml) in 50% ethanol or a buffer appropriate for the experiment in question. In the latter case, bath sonication was required to dissolve the psychosine. At high ethanol concentrations, some inhibition of cytochrome oxidase turnover was seen that was due to the ethanol alone, and the psychosine result was corrected for this. In most experiments the psychosine was allowed to preincubate with the enzyme before the assay was initiated (see the Figure legends). In these cases, the results were always compared with enzyme preincubated at the same temperature in the absence of psychosine. Polarographic (oxygen-consumption) assays were carried out in a Clark or Rank-type oxygen electrode (chamber volume = 3 ml). Corrections were
made for the antioxidation rate in the absence of enzyme. Spectrophotometric (cytochrome c oxidation) assays used a Perkin–Elmer 356 double-beam spectrophotometer in dual-wavelength mode. The wavelength pair used was 550–540 nm and the rate constant calculated by a single exponential fit to these time courses [18]. The rate constant for NNN'N'-tetramethyl-p-phenylenediamine (TMPD) reduction of haem a was measured by adding different concentrations of TMPD to the cyanide-inhibited oxidized enzyme and measuring the increase in haem a reduction at 605–630 nm. Stopped-flow kinetics of haem a reduction by dithionite and cytochrome c were followed at 605 nm on an Applied Photophysics machine. All kinetic assays were carried out at 30 °C. Heps, psychosine, valinomycin, rotenone, ascorbate (L-ascorbic acid, sodium salt), cytochrome c (horse heart type VI) and TMPD dihydrochloride were obtained from Sigma, antimycin and lauryl (dodecyl) maltoside was from Boehringer Mannheim and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was the kind gift of Dr. P. G. Heytler (Du Pont).

RESULTS

Igisu and Nakamura [12] showed that reduced cytochrome c accumulated in rat liver mitochondria in the presence of psychosine, suggesting a possible inhibition of the rate of cytochrome c oxidation. They confirmed this by showing that psychosine inhibited the oxidation of external cytochrome c by the mitochondrial suspension used. However, as cytochrome c should not penetrate intact mitochondria, this inhibition was presumably only seen because of the presence of broken mitochondria in the suspension. We wished to prove that there was a specific interaction of psychosine with cytochrome oxidase, by using the purified enzyme (in this case from bovine heart). Figure 1 shows that the addition of psychosine to active enzyme results in an initial rapid inhibition of turnover (about 50% under these conditions) that is complete within the response time of the oxygen electrode used (10–15 s). There then follows a slower inhibition of the enzyme over the remaining 10–15 min until anaerobiosis occurs.

It is difficult to determine accurately the final extent of inhibition from traces such as Figure 1 for two reasons. The antioxidation rates that must be subtracted to get the corrected enzyme turnover are non-linear with respect to oxygen consumption and, at the low (psychosine-inhibited) rates of oxygen consumption, back diffusion of oxygen into the system becomes significant at low concentrations of oxygen. Therefore the psychosine was preincubated with the enzyme for 15 min and then the reaction was initiated by the addition of cytochrome c to the reaction mixture. In this case, no slow phase of inhibition is observed. Even at relatively high (3 mM) psychosine concentrations, a small (∼10%) enzyme activity remains (Figure 2). It is not possible from this trace to say whether this represents a small population of enzyme molecules that have no psychosine bound or that the psychosine-bound enzyme is 10% active. Similar problems exist with interpreting data from antibody-inhibition studies of cytochrome oxidase [19]. However, the degree of inhibition is similar whether the enzyme is assayed polarographically or spectrophotometrically (Figure 2), suggesting that psychosine is unlikely to be specific kinetic effects on the enzyme/substrate interaction [18] and favouring the idea that the enzyme–psychosine complex is inactive.

The concentration of psychosine required to inhibit enzyme turnover to an equivalent extent is much higher in these experiments than in those by Igisu and Nakamura [12]. Therefore we have directly compared experiments on rat liver mitochondria and purified bovine heart cytochrome oxidase (Figure 3). Using the polarographic assay we again found slow and fast phases of inhibition of mitochondrial respiration by psychosine (results not shown). In order to directly compare our data with those of Igisu and Nakamura, we measured the initial rates of inhibition after psychosine addition. It is clear that equivalent inhibition is achieved at a much lower concentration for the mitochondrial enzyme (although in our hands somewhat higher concentrations are required than used by Igisu and Nakamura for inhibition, even in mitochondria). Again we find a small fraction of activity remaining at high psychosine concentrations (results not shown).

The inhibition of cytochrome oxidase turnover may be due to binding of psychosine to cytochrome oxidase or to cytochrome c. The presence of a slow phase of inhibition allows us to...
Inhibition of cytochrome oxidase by psychosine

Figure 3: Effect of psychosine concentration on cytochrome oxidase turnover in rat liver mitochondria

The initial rate of respiration was measured after the addition of psychosine (relative to the rate before psychosine addition) to actively respiring mitochondria (■). For comparison, the initial rates of inhibition seen with the purified enzyme are also shown (▲). Conditions: mitochondria (0.2 mg/ml protein in 5 mM Tris/HCl, pH 7.4, 150 mM sucrose, 10 mM KCl, 5 mM MgCl₂, 1 mM potassium phosphate with 3.3 mM succinate as respiratory substrate) and purified enzyme (as per polarographic assay in Figure 2).

Figure 4: Effect of preincubation of psychosine with cytochrome c or cytochrome oxidase on the time course of enzyme inhibition

Cytochrome c (▲, 5.7 μM) or cytochrome oxidase (■, 17.5 nM haem a₃) was added to the oxygen electrode chamber in 50 mM potassium phosphate, pH 7.4, 0.1% lauryl maltoside, 5 mM ascorbate, 0.3 mM TMPD. Psychosine (270 μM) was then added and, after the indicated incubation time, the reaction was initiated with the missing component (cytochrome c or cytochrome oxidase as appropriate). The initial rates of steady-state turnover were calculated relative to that for control enzyme preincubated for the same length of time in the electrode chamber in the absence of psychosine.

distinguish between these two possibilities (at least for that slow phase). Preincubation of psychosine with cytochrome c has no effect on the initial inhibition and a slow phase is still observed (Figure 4). However, preincubation of psychosine with cytochrome oxidase increases the initial inhibition and a new, very slow phase that is not specific to psychosine, is observed.

Owing to the species and tissue variability of cytochrome oxidase subunits [20], we wished to compare the effect of psychosine on proteoliposomes and submitochondrial particles from the same source (bovine heart). Studies on the turnover of internally and externally facing enzyme molecules might also allow us to determine the side of the enzyme to which psychosine is bound [21]. Figure 5 shows that psychosine inhibits both internally and externally facing enzyme molecules in proteoliposomes or Keilin–Hartree submitochondrial particles. Thus, it appears that psychosine can either cross the liposomal/mitochondrial membrane, or that it binds to the enzyme within the membrane. This is perhaps not surprising given the hydrophobic nature of the molecule. Again inhibition is more efficient in mitochondrial membranes, suggesting that the differences seen in Figure 3 are not due to tissue or species variability. Most surprisingly this difference appears to be due to the slow interaction of psychosine with the enzyme as, though the proteoliposomes and submitochondrial particles have similar fast phases of inhibition, there is no detectable slow inhibition in the proteoliposomes. Furthermore the inhibition in mitochondrial particles can be relieved by the addition of enzyme-free liposomes, in a similar fashion to the relief of inhibition by high concentrations of serum albumin, either human [12,22] or bovine (results not shown). This effect saturated at about 3 mg/ml added liposomes and only appeared to return activity to about 70% of that before psychosine addition.

These results suggest that the weaker inhibition seen in proteoliposomes and detergent-solubilized enzymes is due to a preference for psychosine to bind to lipids and detergent molecules, rather than cytochrome oxidase. In mitochondrial and submitochondrial particles there is a far higher protein/lipid ratio and the weak interaction of psychosine with the enzyme can predominate. Although these experiments raise the possibility that there may be two specific psychosine-binding sites on the enzyme (one weak and one strong), the biphasic binding may just be a consequence of the kinetics of competition for sites as psychosine enters the oxidase solubilization.

In both the case of submitochondrial particles and proteoliposomes, there was a similar degree of rapid inhibition, whether the internal or external activity was measured. As in the external assay the cytochrome c/cytochrome oxidase ratio is over 500:1, whereas in the internal assay it is close to unity, this suggests that the rapid phase of inhibition cannot be due to psychosine binding to cytochrome c alone as in this case one would expect significantly less inhibition in the external assay. Thus both phases of inhibition must arise from binding to cytochrome oxidase.

Although it is not clear what is the reason for the two phases of psychosine binding to cytochrome oxidase, several factors can be ruled out. Preincubation with the oxidized enzyme results in a similar time course of inhibition to that seen on addition of psychosine to the actively respiring enzyme (compare Figures 1 and 3). Therefore it is not the case that a particular redox intermediate binds psychosine better than any other, as is the case for cyanide or formate binding [7]. The two phases are also not related to the active-site heterogeneity that results in slow ("resting") and fast ("pulsed") forms of the oxidized enzyme [7]; our soluble enzyme is predominantly slow and the mitochondrial enzyme is fast, yet both have a similar biphasic interaction. The ability of soyalecine (soya-bean phospholipid) to titrate out most of the slow phase suggests that the fast phase may be specific binding to this protein and the slow phase could be to less specific binding to the boundary lipids around the complex. Alternatively as the cardiolipin content of soyalecine is not high [23], it may be that the resistant fast phase is due to a specific psychosine/cardiolipin interaction (or to heterogeneity in the cardiolipin content of cytochrome oxidase preparations).

The steady-state kinetics of cytochrome c oxidation by cytochrome oxidase are illustrated in Figure 6. This experiment was
Internally and externally facing cytochrome oxidase activity was assayed in cytochrome \( \text{c}\)-loaded cytochrome oxidase proteoliposomes (\( \text{c}\)-loaded COV) and Kellin–Hartree sub mitochondrial particles (KHP) by the addition of ascorbate/cytochrome \( \text{c} \) (external) and ascorbate/TMPD (internal). Both activities were assayed together by the addition of succinate to KHP. Conditions: in all cases the buffer used was 50 mM potassium phosphate, pH 7.4, and 200 \( \mu \text{M} \) psychosine was added at \( t = 0 \). \( \text{c}\)-loaded COV internal activity (■): vesicles (0.6 mg/ml phospholipid, 30 \( \text{nM} \) haem \( \text{aa}_2 \), 5 mM sodium ascorbate, 1 \( \mu \text{M} \) FCCP, 0.1 \( \mu \text{g/ml} \) valinomycin, 2 mM TMPD). \( \text{c}\)-loaded COV external activity (▲): as for internal activity with 23.5 \( \mu \text{M} \) cytochrome \( \text{c} \) instead of TMPD. KHP internal activity (▲): KHP (0.3 mg/ml protein, 72 \( \text{nM} \) haem \( \text{aa}_2 \), 5 mM sodium ascorbate, 1 \( \mu \text{M} \) antimycin, 1 \( \mu \text{M} \) rotenone, 0.6 mM TMPD). KHP external activity (▲): as for internal activity with 23.5 \( \mu \text{M} \) cytochrome \( \text{c} \) instead of TMPD. KHP internal + external activity (▲): as for internal activity with 23.5 \( \mu \text{M} \) cytochrome \( \text{c} \) + 13.3 mM succinate, 21.3 \( \mu \text{M} \) cytochrome \( \text{c} \) + 1 \( \mu \text{M} \) rotenone. KHP + liposomes (▲): as for internal + external activity + 5 mg/ml liposomes added at \( t = 8 \text{ min} \).

The simplest explanation for this form of behaviour is that psychosine forms a tight complex with a proportion of the enzyme molecules and that this complex is inactive. This is analogous to the case of irreversible inhibition, except that removal of the tightly bound psychosine with serum albumin or liposomes restores enzyme activity.

This non-competitive form of inhibition suggests that, in Figures 2 and 3, the residual enzyme turnover seen at high psychosine levels is due to 10% of enzyme molecules that do not have bound psychosine and are 100% active, rather than that 100% of enzyme molecules have bound psychosine and are only 10% active.

We have found no psychosine inhibition of cytochrome oxidase turnover in the absence of cytochrome \( \text{c} \) (with ascorbate/TMPD as substrate). We therefore tested the effect of psychosine on a variety of intermolecular and intramolecular electron-transfer rates (Table 1). Psychosine does not significantly inhibit electron transfer from dithionite or TMPD to haem \( a \). Indeed there is a slight increase in the haem \( a \) reduction rate by dithionite in the presence of psychosine. Similarly there is no inhibition of the intramolecular electron transfer from haem \( a \) to haem \( a_2 \) on addition of dithionite to slow cytochrome oxidase preparations. The optical spectra of the oxidized and dithionite-reduced enzyme are also unaffected by the presence of psychosine (results not shown).

It therefore appears less likely that psychosine is interfering with the redox centres themselves, and more likely that it affects the interaction of cytochrome \( \text{c} \) with the enzyme. This is shown by the rate of reduction of haem \( a \) by cytochrome \( \text{c} \) in the presence of cyanide (Figure 7). In the presence of psychosine

**Figure 5** Psychosine inhibition of cytochrome oxidase in sub mitochondrial particles and proteoliposomes

**Figure 6** Effect of cytochrome \( \text{c} \) concentration on psychosine inhibition of purified cytochrome oxidase.
The other. Final oxidation and preincubated molecules, one altered. Psychosine [3,6,26]. Environment against a fatty acids, neutral long-chain molecules, with or without (■) psychosine (270 μM final concentration).

There is a marked biphasicity in the reduction rate. The faster rate is similar to that seen in the control (Figure 7a). The slower rate (Figure 7b) under these conditions is inhibited by 32% (±3%, n = 5). Again this is consistent with two populations of enzyme molecules, one that has bound psychosine and one that is unaltered. Those molecules with bound psychosine appear to have an altered interaction with cytochrome c with a diminished rate of electron transfer. Whether there are other effects of psychosine on enzyme turnover remains to be determined.

**DISCUSSION**

There are many non-polar inhibitors of membrane enzymes. The specificity of these interactions for particular enzymes argues against a general mechanism via global perturbations of the lipid environment [3,6,26]. There is growing evidence that there is a relatively non-specific hydrophobic binding site on cytochrome oxidase that allows lipid-soluble inhibitors to interact with the enzyme. In addition to psychosine, these include the antitumour drug adriamycin [4], 11 hydrophobic amide local anaesthetics [5,27], quinacrine [28], six fully reduced flavin analogues [29], neutral long-chain alcohols [3], hydroxylamine, hydradiza, semicarbazide, salicylaldoxamine and ethylxanthate [30]. Non-esterified fatty acids, such as palmitate [6], but not oleate (M. A. Sharpe, C. E. Cooper and J. M. Wrigglesworth, unpublished work), also seem to have specific interactions with the enzyme in the presence of a proton electrochemical potential. What is not clear is whether these effectors act via a direct effect of binding on electron flux or via conformational changes in the enzyme caused by perturbation of the protein-lipid/detergent interface.

Many of these inhibitors have in common either a polar headgroup and/or a free amine group. It is usually the neutral form of the compound that is most effective; protonation of the amines for example can decrease the inhibition [3,31]. It is attractive to postulate that the inhibitor site is close to the cytochrome c-binding site with the lipid-soluble tail binding to a hydrophobic patch and the polar residue(s) interfering with cytochrome c binding [3]. There is evidence for both polar and non-polar areas near the cytochrome c-binding site. Cytochrome c binding to the enzyme is primarily via an electrostatic interaction (usually the cytochrome c molecule is positively charged and the oxidase-binding site negatively charged; [32]). However, there are strong hydrophobic interactions as well, especially in the case of the human enzyme [33]. Some direct evidence in favour of hydrophobic inhibitors interfering with the cytochrome c-binding site comes from the competitive inhibition kinetics of many of these inhibitors with respect to cytochrome c concentration [4,5,26–28,31,34]. However, care must be taken with these interpretations as the inhibition kinetics are frequently more complex and vary with the phospholipid/detergent environment [3,27,34].

How does psychosine fit in with this pattern of inhibition? It contains both a primary amine (as do the general anaesthetics) and a polar sugar head group. Its binding is slower than many of the other inhibitors. However, unlike Fe3+-adriamycin [4], this slow inhibition is not a result of a slow permanent inactivation of the oxidase, as the inhibited complex is re-activated by the addition of serum albumin or liposomes. We visualize the psycosine interaction as similar to those of antibodies which bind slowly, but with low off constants, such that there is no appreciable dissociation of the psychosine–enzyme complex in the time course of the steady-state kinetic assay [20]. If this complex is inactive, this will result in the same kinetics as for irreversible inhibition. Further evidence for the parts of the psychosine molecule that are responsible for the interaction with cytochrome oxidase will come from studies of closely related structures (e.g. that lack the amine and/or have different sugar structures).

How relevant are studies of the effect of psychosine on mitochondrial cytochrome oxidase to the aetiology of Krabbe's disease? There is a general acceptance that the accumulation of psychosine due to a primary defect in galactosylceramidase I is the toxic agent in the disease [35]. The primary morphological signs of the disease are the destruction of the oligodendroglial cells from which myelin tissue is formed [36]. This leads to a general depletion of all lipids (especially glycolipids) in the white matter. Recent work has suggested the psychosine inhibition of protein kinase C as a possible primary effect [10]. However, in the twitcher mouse (a model of Krabbe's disease) there is no detectable decrease in protein kinase C activity in the brain cytosol [37]. Psychosine inhibits the incorporation of galactose into myelin-associated lipids (such as cerebroside and sulphatide) in oligodendrogial, but not astroglial, cell cultures [38]. Given the requirement for galactosyl-lipids in myelin it appears likely that this inhibition of lipid metabolism may be the primary cause of demyelination.

However, the large increase in psychosine concentration in the disease (from being undetectable to as much as 0.14 mM [10]) is likely to have important secondary effects on enzymes such as protein kinase C [10], CTP:phosphocholine cytidylyltransferase [11] and cytochrome oxidase. This is especially so given that...
accumulation of psychosine is not confined to the brain [35] and that in vivo the concentrations in the lipid environment where these enzymes are active is likely to be even higher. The studies on proteoliposomes and submitochondrial particles suggest that psychosine is unlikely to be easily compartmentalized in one particular membrane in the cell and that if high concentrations occur they will inevitably inhibit these enzymes. Indeed, when added to neuronal cell cultures, psychosine induces mitochondrial swelling [39]. In this context it is worth noting that genetic deficiencies of cytochrome oxidase can cause a wide variety of neurological symptoms [1].

Possible (non-genetic) treatments for Krabbe’s disease are likely to focus on ameliorating the toxic effects of psychosine or removing psychosine from its site of action. Suggested agents for the former include phosphor ester or dimethyl sulphoxide [40]. However, owing to the multifaceted toxicity of psychosine, it might be better to attempt to bind it in an inactive form. The results of this work clearly show that the phospholipid and protein environment around a membrane enzyme greatly affects its inhibition by psychosine. A similar relief of haemolysis by psychosine is seen on addition of cholesterol [9]. It was postulated that this was due to a complex of psychosine and cholesterol. The resistance of non-brain tissues to the effects of psychosine may be partly due to their larger concentration of cholesterol (and in later stages of myelin breakdown the cholesterol present in the brain seems to be predominantly in the form of cholesterol esters [41]). Thus modulation of the cholesterol and other lipid content in the brain (e.g. cardiolipin) may be another possible therapeutic avenue to explore.

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