Glycerol 3-phosphate dehydrogenase of boar spermatozoa: inhibition by α -bromohydrin phosphate

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Boar spermatozoa incubated with glycerol 3-phosphate as substrate produced CO_2 and an accumulation of dihydroxyacetone phosphate and fructose-1,6-bisphosphate. The rate of oxidation of glycerol 3-phosphate was decreased, as was the production of CO_2 and the two glycolytic intermediates, in the presence of (*R,S*)- α -bromohydrin phosphate. In the presence of inhibitors of stage two of the glycolytic pathway, CO_2 production was prevented, there was a marked increase in the concentration of the glycolytic intermediates but the rate of metabolism of the substrate was unaffected. Oxygen consumption by spermatozoa incubated with glycerol 3-phosphate was unaffected in the presence of rotenone, whereas it was decreased when lactate was offered as the substrate. The results reported here confirm that in boar spermatozoa glycerol 3-phosphate dehydrogenase is an FAD-linked enzyme that is inhibited by (*R,S*)- α -bromohydrin phosphate in, possibly, a competitive manner.

Introduction

The male antifertility agent (*S*)- α -chlorohydrin (3-chloropropan-1,2-diol: I in Fig. 1) is oxidized within the spermatozoa of susceptible species to (*S*)-3-chlorolactaldehyde (II in Fig. 1) which inhibits the activities of triosephosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase, thereby preventing the cells from synthesizing ATP which is essential for the maintenance of motility (Cooney and Jones, 1988). Early biochemical studies with the racemate, (*R,S*)- α -chlorohydrin, led to the proposal that the active metabolite was the phosphorylated derivative, α -chlorohydrin-1-phosphate (III in Fig. 1), although this was not supported by experimental evidence (Mohri *et al.*, 1975). During the course of our investigations into the metabolic activity of boar spermatozoa, we have examined the possible effects of (*R,S*)- α -chlorohydrin-1-phosphate and, while confirming that the compound had no action on the metabolism of fructose, glycerol or lactate (Jones and Chantrill, 1989), observed that it did inhibit the oxidation of glycerol 3-phosphate.

This apparent anomaly, the inhibition of the oxidation of glycerol 3-phosphate without affecting the metabolism of glycerol, was partially explained when it was shown that glycerol was metabolized, not by its conversion to glycerol 3-phosphate, but as glycerol $\rightarrow$ glyceraldehyde $\rightarrow$ glyceraldehyde 3-phosphate (Jones *et al.*, 1992). Attempts to study the action of (*R,S*)- α -chlorohydrin-1-phosphate on the metabolism of glycerol 3-phosphate were hindered by the presence of wide-range phosphatase activity in the samples of boar spermatozoa being used which converted (*S*)- α -chlorohydrin-1-phosphate to (*S*)- α -chlorohydrin, thereby allowing the formation of (*S*)-3-chlorolactaldehyde (Porter and

Jones, 1995). To overcome this, we have synthesized (*R,S*)- α -bromohydrin-1-phosphate and confirmed that this compound also inhibited the metabolism of glycerol 3-phosphate without affecting that of any other substrate since its hydrolysis, to (*R,S*)- α -bromohydrin, produces a compound that has no action on spermatozoa either *in vivo* (Banik *et al.*, 1972) or *in vitro* (Jones and Porter, 1995). The use of 3-chloro-1-hydroxypropanone (Jones *et al.*, 1986) and 3-bromopyruvate (Jones *et al.*, 1995), which are specific inhibitors of stage two of the glycolytic pathway in boar spermatozoa, has enabled us to determine that (*R,S*)- α -bromohydrin-1-phosphate inhibits glycerol 3-phosphate dehydrogenase in what appears to be a competitive manner and that the enzyme is FAD-linked and probably bound to the mitochondrial membrane.

Materials and Methods

Substrates and chemicals

L-[U- ^{14}C]Glycerol 3-phosphate, [U- ^{14}C]glycerol, D-[U- ^{14}C]fructose and L-[U- ^{14}C]lactate were purchased from

Fig. 1. The structures of (*S*)- α -chlorohydrin (I), (*S*)-3-chlorolactaldehyde (II), (*R,S*)- α -chlorohydrin phosphate (III, X = Cl) and (*R,S*)- α -bromohydrin phosphate (III, X = Br).

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Amersham International plc (Amersham, Bucks). All enzymes, substrates and co-factors were obtained either from Sigma Chemical Co. (St Louis, MO) or Boehringer Mannheim Australia Pty Ltd (Castle Hill, NSW). All other chemicals and reagents were of analytical grade and all solutions were prepared in water purified by reverse osmosis.

3-Chloro-1-hydroxypropanone was prepared from 3-chloro-1-hydroxypropanone dimethyl acetal by the procedure of Jones *et al.* (1986). Neutralized solutions of chlorohydroxypropanone (10 mmol l^{-1}) were prepared immediately before use and added to incubations at a final concentration of 0.5 mmol l^{-1} . (*R,S*)- α -Bromohydrin phosphate biscyclohexylamine salt was synthesized by the method of Mashford and Jones (1978); recrystallization from dioxane gave the compound as white crystals, m.p. $148\text{--}150^\circ\text{C}$ (reported m.p. (Fondy *et al.*, 1970) $124\text{--}127^\circ\text{C}$). 3-Bromopyruvate hydrate, purchased from Aldrich Chemical Co. (Milwaukee, WI), was recrystallized several times from dry chloroform, m.p. $74\text{--}77^\circ\text{C}$ (reported m.p. (Sprinson and Chargaff, 1946) 74°C). Solutions were neutralized with solid NaHCO_3 and the compound added to incubates as sodium bromopyruvate at a final concentration of 0.5 mmol l^{-1} .

Metabolic studies

Cauda epididymides, excized from the testis-epididymis complex of mature boars (*Sus scrofa*; various crosses of Duroc, Hampshire, Landrace and Large White) 15–20 min after slaughter at the abattoir, were placed immediately in Dewar flasks containing PBS (Stevenson and Jones, 1985) at 34°C . The sealed flasks were transported to the laboratory where the spermatozoa were flushed from the epididymides with PBS (34°C) from incisions made in the third and fourth segments of the cauda according to the classification of Holtz and Smidt (1976). The spermatozoa were washed and prepared as a 10% (v/v) suspension in PBS at 34°C according to our established procedure (Jones and Chantrill, 1989). Each suspension, containing spermatozoa from 10–15 boars, contained $15\text{--}20 \text{ mg protein ml}^{-1}$ representing of the order of $10^7 \text{ cells ml}^{-1}$.

Incubations were performed in standard Warburg flasks shaken at $120 \text{ oscillations min}^{-1}$ at 34°C with air as the gas phase. Incubations (1 ml) comprised substrate ($50 \mu\text{l}$, 200 nCi if radioactive), inhibitor or PBS ($50 \mu\text{l}$) and were started with the addition of the 10% sperm suspension ($900 \mu\text{l}$). Incubations were terminated by the addition of $3 \text{ mol HClO}_4 \text{ l}^{-1}$ ($100 \mu\text{l}$) and the deproteinized, neutralized and desalted incubation solutions used for the assays of substrates and products. Time-course incubations were performed in open 25 ml conical flasks and aliquots (1 ml) were taken at specified periods and added to $3 \text{ mol HClO}_4 \text{ l}^{-1}$ ($100 \mu\text{l}$). Zero-time samples were removed approximately 10–15 s after preparation of the incubation suspensions. Assays for metabolically derived CO_2 (Stevenson and Jones, 1982), fructose-1,6-bisphosphate and dihydroxyacetone phosphate (Michal and Beutler, 1975), glycerol 3-phosphate and lactate (Lang, 1985) were performed according to the literature. Protein was estimated (Lowry *et al.*, 1951) using BSA as a standard.

Suspensions of spermatozoa (10% v/v, 10 ml) were centrifuged at $2000 g$ for 5 min at 4°C . The supernatant

solutions were discarded; the pellets suspended in sucrose (0.25 mmol l^{-1} , 1.25 ml) containing EDTA (1 mmol l^{-1}) and mercaptoethanol (1 mmol l^{-1}); and the cells disrupted by shaking with Triton X-100 (1%, 0.14 ml). The suspensions were centrifuged at $20\,000 g$ for 10 min at 4°C and the supernatant solutions assayed for NAD^+ -dependent glycerol 3-phosphate dehydrogenase activity (Lang, 1985).

The oxygen consumption of sperm suspensions was measured using an oxygen electrode (Model 10, Rank Bros, Cambridge) calibrated with air-saturated water ($240 \mu\text{mol O}_2 \text{ l}^{-1}$ at 34°C) (Umbreit *et al.*, 1957). Sperm suspensions (5% (v/v), $900 \mu\text{l}$) were placed in the reaction chamber and vigorously stirred at 34°C . Substrates and inhibitors ($50 \mu\text{l}$) were added at final concentrations of 0.5 mmol l^{-1} (fructose), 1 mmol l^{-1} (glycerol 3-phosphate and lactate), $700 \mu\text{mol l}^{-1}$ (rotenone), 0.25 mmol l^{-1} (3-bromopyruvate and α -bromohydrin phosphate).

Preliminary inhibition studies were performed by incubating sperm suspensions for 20 min in the presence of α -bromohydrin phosphate (0, 5 and 10 mmol l^{-1}) and a range of concentrations of L-glycerol 3-phosphate ($1\text{--}10 \text{ mmol l}^{-1}$). The fall in the concentration of glycerol 3-phosphate over this period was equated to the velocity of the reaction.

Statistical analyses

Values are presented as the mean $\pm$ SEM for the number (*n*) of experiments performed. Significance of difference was determined by an unpaired two-tailed *t* test.

Results

Boar spermatozoa oxidatively metabolized fructose, glycerol, glycerol 3-phosphate and lactate to CO_2 , but dihydroxyacetone phosphate and fructose-1,6-bisphosphate accumulated only from glycerol 3-phosphate and this was accompanied by comparatively less production of CO_2 (Table 1). The production of CO_2 from fructose, glycerol and lactate was unaffected by the presence of α -bromohydrin phosphate (5 mmol l^{-1}), but that from glycerol 3-phosphate was inhibited, the rate of metabolism of the substrate decreased (Fig. 2), and the concentrations of dihydroxyacetone phosphate and fructose-1,6-bisphosphate were concomitantly lower (Fig. 3).

In the presence of 3-chloro-1-hydroxypropanone (0.5 mmol l^{-1}) or 3-bromopyruvate (0.5 mmol l^{-1}), the oxidation of glycerol 3-phosphate to CO_2 was almost completely inhibited but neither compound had any appreciable effect on its rate of disappearance (Fig. 4). Unlike the effect of α -bromohydrin phosphate, both chlorohydroxypropanone and 3-bromopyruvate resulted in an increase in the concentrations of dihydroxyacetone phosphate and fructose-1,6-bisphosphate (Fig. 5).

The maximum NAD^+ -dependent glycerol 3-phosphate dehydrogenase activity in boar spermatozoa was determined to be such that it was able to convert approximately 700 pmoles of glycerol 3-phosphate to dihydroxyacetone phosphate $\text{min}^{-1} \text{ mg}^{-1} \text{ protein}$ ($n = 6$). Thus, operating at maximum activity for 30 min, approximately 20 nmoles of substrate could theoretically be oxidized in incubation

Table 1. The production of CO₂, dihydroxyacetone phosphate, fructose-1,6-bisphosphate and lactate by boar spermatozoa incubated at 34°C for 1 h with different substrates

Substrate	<i>n</i>	CO ₂	Dihydroxyacetone phosphate	Fructose-1,6-bisphosphate	Lactate
Glycerol 3-phosphate (2 mmol l ⁻¹)	13	30.0 ± 1.7	21.0 ± 2.1	7.8 ± 0.8	25.0 ± 2.4
Fructose (1 mmol l ⁻¹)	8	75.0 ± 8.9	0.4 ± 0.1	0.1 ± 0.1	44.2 ± 5.8
Glycerol (2 mmol l ⁻¹)	7	62.1 ± 11.2	1.0 ± 0.2	0.3 ± 0.1	51.1 ± 5.7
Lactate (2 mmol l ⁻¹)	4	110 ± 1	0.3 ± 0.1	0	50.1 ± 6.3

Values are nmoles per mg protein.

Fig. 2. Effect of α -bromohydrin phosphate on (a) the oxidation of glycerol 3-phosphate to CO₂ ($n=9$) and (b) the consumption of glycerol 3-phosphate ($n=11$) by boar spermatozoa. Spermatozoa were incubated at 34°C for 1 h with glycerol 3-phosphate (2 mmol l⁻¹) in the presence (●) or absence (○) of (*R,S*)- α -bromohydrin phosphate (5 mmol l⁻¹).

suspensions, whereas over 90 nmoles of glycerol 3-phosphate were oxidized min⁻¹ mg⁻¹ protein over the same period (Fig. 4).

Fig. 3. Effect of α -bromohydrin phosphate on the concentration of (a) dihydroxyacetone phosphate (DHAP) ($n=8$) and (b) fructose-1,6-bisphosphate (F-1,6-P₂) ($n=8$) in boar spermatozoa. Spermatozoa were incubated at 34°C for 1 h with glycerol 3-phosphate (2 mmol l⁻¹) in the presence (●) or absence (○) of (*R,S*)- α -bromohydrin phosphate (5 mmol l⁻¹).

The consumption of oxygen by sperm suspensions, determined as 0.67 ± 0.20 nmol min⁻¹ mg⁻¹ protein ($n=5$), was reduced to 0.17 ± 0.05 nmol min⁻¹ mg⁻¹ protein ($n=6$) in

Fig. 4. Consumption of glycerol 3-phosphate by boar spermatozoa (○) ($n=15$). Spermatozoa were incubated at 34°C for 1 h with glycerol 3-phosphate (2 mmol l^{-1}) in the presence of (●) 3-chloro-1-hydroxypropanone (0.5 mmol l^{-1}) ($n=14$) or (■) 3-bromopyruvate (0.5 mmol l^{-1}) ($n=5$).

the presence of 3-bromopyruvate, which inhibited the oxidation of endogenous substrates. With glycerol 3-phosphate as the substrate, the consumption of oxygen increased to $1.53 \pm 0.07\text{ nmol min}^{-1}\text{ mg}^{-1}\text{ protein}$ ($n=9$); this was decreased to $1.23 \pm 0.09\text{ nmol min}^{-1}\text{ mg}^{-1}\text{ protein}$ ($n=6$) on addition of 3-bromopyruvate and fell further to 0.59 ± 0.16 ($n=4$) on the addition of α -bromohydrin phosphate. Spermatozoa consumed more oxygen with glycerol 3-phosphate as the substrate than with either fructose or lactate, and rotenone ($700\text{ }\mu\text{mol l}^{-1}$) inhibited the uptake of oxygen from lactate but not from glycerol 3-phosphate (Fig. 6).

When sperm suspensions were incubated in the presence of α -bromohydrin phosphate (5 and 10 mmol l^{-1}), inhibition of the rate of disappearance of glycerol 3-phosphate was overcome as the concentration of substrate was increased.

Discussion

Inhibition by α -bromohydrin phosphate of the oxidation to CO_2 of glycerol 3-phosphate, but not that of fructose, glycerol or lactate, indicates that the action of the compound involves the conversion of glycerol 3-phosphate to dihydroxyacetone phosphate. Interference with this process would account for the decrease in the production of CO_2 and dihydroxyacetone phosphate and in the consumption of glycerol 3-phosphate. Incubations performed with glycerol 3-phosphate as the substrate in the presence of either 3-chloro-1-hydroxypropanone, which inhibits triosephosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase (Jones and Cooney, 1987, Cooney and Jones, 1988), or 3-bromopyruvate, which inhibits glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate kinase (Jones *et al.*, 1995), also prevented the production of CO_2 , resulted in an increase in concentrations of dihydroxyacetone phosphate and fructose-1,6-bisphosphate, but had no appreciable effect on the

Fig. 5. Effect of 3-bromopyruvate on the concentration of (a) dihydroxyacetone phosphate (DHAP) ($n=6$) and (b) fructose-1,6-bisphosphate (F-1,6- P_2) ($n=6$) in boar spermatozoa. Spermatozoa were incubated at 34°C for 1 h with glycerol 3-phosphate (2 mmol l^{-1}) in the presence (●) or absence (○) of 3-bromopyruvate (0.5 mmol l^{-1}).

consumption of glycerol 3-phosphate. These results confirm that glycerol 3-phosphate was being oxidized to dihydroxyacetone phosphate and that α -bromohydrin phosphate inhibited the rate of this oxidation.

Glycerol 3-phosphate can be oxidized to dihydroxyacetone phosphate by either an NAD^+ -dehydrogenase (EC 1.1.1.8) or an FAD-dependent (EC 1.1.99.5) glycerol 3-phosphate dehydrogenase (Voet and Voet, 1995); the former is located in the cytoplasm whereas the latter is bound to the inner mitochondrial membrane (Klingenberg and Bucholz, 1970). Examination of the activity of an NAD^+ -dependent glycerol 3-phosphate dehydrogenase in boar spermatozoa revealed that its activity was extremely low and, even when operating at the maximum activity that could be assayed, could not account for the rate of consumption of glycerol 3-phosphate. The consumption of oxygen when glycerol 3-phosphate was the

Fig. 6. Oxygen uptake by boar spermatozoa incubated with (A): glycerol 3-phosphate (■), fructose (▨) or lactate (▤); (B): glycerol 3-phosphate (■) and glycerol 3-phosphate plus rotenone (□) ($P < 0.05$) and (C): lactate (▨) and lactate plus rotenone (□) ($P < 0.0001$). Boar sperm suspensions were stirred at 34°C and the oxygen consumption measured for 10 min ($n = 4$).

substrate was greater than when fructose or lactate were the substrates and this indicates that the oxidation of glycerol 3-phosphate is rapid, very dependent on the presence of oxygen and is inhibited by α -bromohydrin phosphate.

The conversion of fructose or glucose to pyruvate produces ATP, by substrate-level phosphorylation, and NADH. It has been suggested that boar spermatozoa re-oxidize this NADH to NAD^+ to convert the pyruvate to lactate and that it is lactate which is the main metabolic fuel for mitochondrial metabolism (Jones and Chantrill, 1989). Thus, both fructose and lactate ultimately transfer cytoplasmic reducing equivalents to the mitochondria as NADH and these are processed by electron transport. An FAD-linked glycerol 3-phosphate dehydrogenase, however, would also transfer its reducing equivalents to the electron transport chain but at a position beyond that of NADH dehydrogenase. Inhibition of electron transport between the entry of NADH and FADH_2 by rotenone showed little effect on the oxidation of glycerol 3-phosphate but had a marked effect on that of lactate.

These results confirm those of Ford (1981) that, in boar spermatozoa, glycerol 3-phosphate is oxidized primarily by an FAD-dependent dehydrogenase that is probably extrinsically bound to the inner mitochondrial membrane (Klingenberg and Bucholz, 1970). In this respect, boar spermatozoa are similar to those of rams (Ford, 1981), rats (Ford, 1981; Brooks, 1978; Schenkman *et al.*, 1965) and cocks (Yamada and Terada, 1974) but not those of mice (Burgos *et al.*, 1982) or humans (Ford, 1981). The ability of α -bromohydrin phosphate to inhibit this enzyme in boar spermatozoa is probably due to the *R*-isomer which has identical stereochemistry to the normal substrate (*S*)- (or *sn*-) glycerol 3-phosphate. The manner of inhibition appears to be competitive since preliminary studies showed that increasing the concentration of substrate overcame the inhibitory action of a constant concentration of α -bromohydrin phosphate.

Given that boar spermatozoa are efficient at metabolising glycerol 3-phosphate (Jones and Gillan, 1996), and that lactate, rather than pyruvate, is the major mitochondrial fuel (Jones and Chantrill, 1989), the presence of a membrane-bound glycerol 3-phosphate dehydrogenase makes it unnecessary to possess other shuttle mechanisms to transfer cytoplasmic reducing equivalents to the mitochondria. An FAD-linked glycerol 3-phosphate dehydrogenase acts, in effect, as the glycerol 3-phosphate shuttle while the entry of cytoplasmic lactate into the mitochondria performs a similar task by transferring cytoplasmic-derived reducing equivalents directly to the electron transport chain (Calvin and Tubbs, 1978; Gallina *et al.*, 1994). Thus the metabolic activity of the cell would be most efficiently directed towards the mitochondrial production of ATP for cell motility.

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