Properties and regulation of a trans-plasma membrane redox system in rat liver

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1. Regulation of the reduction of ferricyanide by the isolated perfused rat liver was studied. 2. The rate of reduction was dependent on the rate of supply of ferricyanide and independent of perfusate oxygen concentration. 3. The effect of pH was also examined; the rate of reduction was optimal at pH 7.4 and was inhibited to a greater extent by pH values below 7.4 than those above 7.4. 4. The effects of substrates on the rate of ferricyanide reduction was assessed. Reductants of the cytosolic and mitochondrial NADH/NAD⁺ couple were tested. 2-Hydroxybutyrate (10 mM), lactate (10 mM), glycerol (10 mM) and ethanol (10 mM) each had no effect. Dihydroxyacetone (10 mM) stimulated the rate. 5. Dehydroascorbate (1 mM), stimulated the rate of ferricyanide reduction; the stimulation did not appear to be attributable to the production of reduced substances that were excreted to reduce extracellular ferricyanide. 6. The effects of glucagon and cyclic AMP on the rate of ferricyanide reduction were examined. Glucagon inhibited the rate by approx. 30% and half-maximal inhibition occurred at 0.1 nM, corresponding to the concentration at which half-maximal stimulation of glucose release occurred. Cyclic AMP stimulated glucose release but had no significant effect on the rate of ferricyanide reduction. It is concluded that the trans-plasma membrane redox system of liver that reduces extracellular ferricyanide is regulated by glucagon. The rate is also altered by the substrate dihydroxyacetone. The effect of glucagon may be direct as it cannot be mimicked by cyclic AMP and it occurs directly following exposure to the hormone.

In a previous paper we have presented evidence for the extracellular reduction of ferricyanide by perfused liver and isolated rat hepatocytes (Clark *et al.*, 1981). In that work a high-affinity activity for ferricyanide reduction was noted that was unaffected by rotenone, antimycin A, KCN, NaN₃ or *p*-hydroxymercuribenzoate but was inhibited by 2.6 mM-CaCl₂, 2-heptyl-4-hydroxyquinoline-*N*-oxide and ferrocyanide. The observations were consistent with the occurrence of a trans-plasma membrane redox system of liver that reduced extracellular ferricyanide to ferrocyanide. Since the properties of the reduction process were similar to those of the NADH:ferricyanide oxidoreductase found in isolated liver plasma membranes (L6w & Crane, 1978) it was relevant in the present study to examine in detail some of the regulatory properties of the process. To avoid complications introduced by the mitochondrial NADH-oxidoreductases pre-

sent in hepatocyte preparations (e.g. see Clark *et al.*, 1981), the present experiments were confined to the perfused liver system.

Materials and methods

Materials

D-Glucose, sodium pyruvate, L(+)-lactic acid, DL-2-hydroxybutyrate, dihydroxyacetone, D-fructose, glycerol, cyclic AMP, and dibutyl cyclic AMP were purchased from Sigma. Other reagents were analytical grade from BDH. Potassium ferricyanide was converted to sodium ferricyanide by passage through a column of cation-exchange resin. Dehydroascorbate was purified by recrystallization of commercially available material from glacial acetic acid/HCl (Staudinger & Weis, 1964). Glucagon was generously given by Dr. W. Bromer of Eli Lilly.

Liver perfusion

Livers of fed rats (170–250g body wt.) were perfused at 37°C with 700ml of Krebs–Henseleit bicarbonate buffer containing 1.3mM-CaCl₂ and 0.5mM-sodium ferricyanide and which was equilibrated with O₂/CO₂ (19:1). After 5min of non-recirculating perfusion to remove blood cells, the perfusion was changed to a recirculating mode (total volume 500ml). The pH of the perfusate was maintained between 7.38 and 7.42 by adding dilute acid or base as required. Unless indicated otherwise the perfusate flow rate was 40ml/min and livers weighed between 7 and 10g.

The change in concentration of ferricyanide was determined by monitoring the decrease in A_{420} of the perfusate. Samples (1.2ml) were removed at 2min intervals into ice-cold tubes. After all of the samples had been collected the tubes were centrifuged for 2min at 8000g and the A_{420} was read. To minimize reduction of ferricyanide in the absence of the liver, the apparatus was cleaned with 0.01M-NaOH between perfusions.

Results and discussion

We have previously shown that extracellular ferricyanide is reduced to ferrocyanide by the perfused rat liver (Clark *et al.*, 1981). Thus the present study was conducted to examine factors which may operate to control this process. Fig. 1 shows that the rate of ferricyanide reduction was independent of oxygen concentration. However, if the perfusate flow rate was decreased to less than 40ml/min the rate of reduction of ferricyanide was also decreased. Calculations based on the rate of ferricyanide reduction at 40ml/min of 0.20μmol/min per g of liver indicate that if the flow rate was 5ml/min, in each min approx. 40% of the available ferricyanide in the perfusate within the liver would be reduced. Since the apparent K_m for ferricyanide is 0.13mM (Clark *et al.*, 1981) a decrease in ferricyanide concentration from 0.5 to 0.3mM would be expected to significantly inhibit the rate of reduction. The possible accumulation of inhibitory concentrations of ferrocyanide (Clark *et al.*, 1981) may also inhibit the rate of ferricyanide reduction.

The data of Fig. 1 imply that the trans-plasma membrane redox process is not dependent on cell viability or the maintenance of normal energy status. At low flow rates (less than 10ml/min) the hepatic loss of lactate dehydrogenase was not altered but the hepatic content of ATP decreased from 2.85 ± 0.35 to 1.57 ± 0.19μmol/g fresh wt. of liver (mean ± S.E.M. for three livers). Thus the possibility that the reduction of ferricyanide is ATP-dependent seems unlikely. For red blood cells a link has been established between extracellular ferricyanide and intracellular ATP (Manyai & Szekely, 1954) but the

Fig. 1. Effect of perfusate flow rate (a) and oxygen tension (b) on the rate of reduction of ferricyanide by the isolated perfused rat liver

Livers of fed rats were perfused as described in the Materials and methods section. The initial concentration of ferricyanide was 0.5mM. The perfusate flow rate (●) was set at the value shown for the duration of the perfusion. The average linear rates over 40min are given. The percent saturation with oxygen (○) was achieved by gassing with O₂/CO₂ (19:1) or with N₂/CO₂ (19:1) or by using a mixture of the two. Where appropriate mean values ± S.E.M. from the perfusions have been calculated. Other values are the mean from two perfusions.

processes involving ferricyanide reduction were not shown to be ATP-dependent. Indeed, these workers (Manyai & Szekely, 1954) observed that the addition of ferricyanide to the medium of erythrocyte suspensions induced ATP synthesis inside the cells. Since the ferricyanide did not penetrate into the cells (Szekely *et al.*, 1952), it was concluded that the oxidizing agent affected ATP synthesis without direct contact with the glycolytic system. These observations were later confirmed and extended (Passow, 1963) to show that ferrocyanide, in contrast with ferricyanide, induced ATP break-

down. In the present study the hepatic content of ATP was found to be 2.85 ± 0.35 (3) $\mu\text{mol/g}$ fresh wt. of liver after perfusion for 40 min with 0.5 mM-ferricyanide. Since the control (no ferricyanide) value was 3.3 ± 0.38 (3) $\mu\text{mol/g}$ fresh wt. of liver it appeared that the hepatic content of ATP was not significantly affected by ferricyanide. In addition the rate of gluconeogenesis from 1.3 mM-lactate was unaffected by ferricyanide; the rates at 0, 0.5, 0.85, 1.0 and 2.0 mM-ferricyanide were 0.045, 0.044, 0.042, 0.041 and 0.043 $\mu\text{mol/min per g wet wt. of liver}$, respectively. Finally the data of Fig. 1 also imply that oxygen was not directly involved in the intracellular reduction of ferricyanide.

In Fig. 2 the effect of perfusate pH on the rate of reduction of ferricyanide was examined. The pH optimum was 7.4. In general, the rate was less affected by pH values above 7.4 than below 7.4. These data are consistent with the view that reduction of extracellular ferricyanide may be accompanied by proton extrusion from the cell into the extracellular fluid. Thus at pH values below neutrality the rate is more strongly inhibited. Proton extrusion accompanying ferricyanide reduction has been reported for yeast and plant cells (Crane *et al.*, 1981; Craig & Crane, 1981).

In order to gain an insight into the function of the apparent trans-plasma membrane redox activity and, as well, to identify the source of reducing equivalents for the reduction of ferricyanide, various substrates were tested (Table 1). These included substrates that reduce either the mitochondrial or cytosolic compartments of the pyridine nucleotides.

Fig. 2. Effect of perfusate pH on the rate of reduction of ferricyanide by the isolated perfused rat liver
Livers of fed rats were perfused as described in the Materials and methods section. The initial concentration of ferricyanide was 0.5 mM. The pH of the perfusate was maintained at the value shown for the duration of the perfusion. The average linear rates over 40 min are given. Each result is the mean of two perfusions.

Table 1. Effects of substrates on the rate of ferricyanide reduction by the perfused rat liver

Livers were perfused as described in the Materials and methods section. The ferricyanide concentration was 0.5 mM. Perfusions were conducted in a recirculating mode for a total of 40 min. Substrates were added at 20 min. The initial (0–20 min) and the final (20–40 min) rates were determined. Mean values $\pm$ s.e.m. have been calculated and the number of perfusions are given in parentheses. Paired Student's *t* test was applied to assess the significance of the experimental situation versus the relevant control: *, *P* < 0.05; n.s., not significant.

Addition (final concn.) at 20 min	Ferricyanide reduction rate ($\mu\text{mol/min per g wet wt. of liver}$)	
	Before addition (0–20 min)	After addition (20–40 min)
None	0.203 $\pm$ 0.008 (5)	0.200 $\pm$ 0.008 (5)
Glucose (10 mM)	0.193 $\pm$ 0.018 (3)	0.184 $\pm$ 0.019 (3) n.s.
Pyruvate (10 mM)	0.180 $\pm$ 0.011 (3)	0.182 $\pm$ 0.011 (3) n.s.
Ethanol (10 mM)	0.203 $\pm$ 0.004 (3)	0.192 $\pm$ 0.009 (3) n.s.
Lactate (10 mM)	0.195	0.187 (2) n.s.
2-Hydroxybutyrate (10 mM)	0.236 $\pm$ 0.017 (3)	0.215 $\pm$ 0.024 (3) n.s.
Dihydroxyacetone (10 mM)	0.185 $\pm$ 0.017 (5)	0.223 $\pm$ 0.015 (5) *
Glycerol (10 mM)	0.201 (2)	0.184 (2)
Fructose (2 mM)	0.210 $\pm$ 0.017 (3)	0.187 $\pm$ 0.016 (3) n.s.

2-Hydroxybutyrate, ethanol, glycerol and lactate were without effect. In addition, substrates such as pyruvate and fructose that lead to oxidation of pyridine nucleotides of the cytosol had no effect. Indeed, the only substrate to alter the rate of ferricyanide reduction was dihydroxyacetone (10mM) which was stimulatory. Since 2-hydroxybutyrate could be expected to lead to reduction of the mitochondrial compartment of pyridine nucleotides and lactate and ethanol to lead to reduction of the cytosolic compartment of pyridine nucleotides, the present data imply that reduction of either compartment does not favour reduction of extracellular reduction of ferricyanide. These findings are different to those observed with yeasts, where ferricyanide reduction was enhanced by ethanol (Crane *et al.*, 1981).

The basis for the stimulation produced by dihydroxyacetone is not clear. Dihydroxyacetone is metabolized by the liver to produce glucose as well as lactate and pyruvate (Clark *et al.*, 1974). Orringer & Roer (1979) have argued that ferricyanide reduction in human erythrocytes occurs as a result of NADH oxidation. They further propose that NADH generated at glyceraldehyde 3-phosphate dehydrogenase is the primary source of reducing equivalents for this reduction. Thus substrates such as fructose, dihydroxyacetone and glycerol, which lead to increased flux through glyceraldehyde 3-phosphate dehydrogenase, might be expected to lead to increased production of NADH from this step and to increased ferricyanide reduction. In general, this was not the case, as dihydroxyacetone was the only one of the three above substrates that increased the rate of ferricyanide reduction (Table 1).

Dehydroascorbate (0.1–1 mM) increased the rate of ferricyanide reduction by perfused liver in a similar manner to that observed by Orringer & Roer (1979) using human erythrocytes (Fig. 3). These workers argued that dehydroascorbate increases ferricyanide reduction by crossing the cell membrane to be reduced to ascorbate, which then left the cell to react directly with ferricyanide. For liver, this possibility seems unlikely since ferricyanide-reducing substrates were not produced during perfusion with dehydroascorbate. Thus the observed increase in rate of ferricyanide reduction produced by dehydroascorbate may arise either as a direct effect on ferricyanide reduction or from a catalytic role involving intracellular oxidation and reduction.

Isolated plasma membranes from mouse and rat liver have been shown to possess NADH oxidoreductase activity that is stimulated by glucagon (Löw & Crane, 1976; Goldenberg *et al.*, 1978). Thus it was pertinent in the present study to examine in detail the effects of glucagon on the rate of ferricyanide reduction in the perfused rat liver. In Fig. 4(a) the dose-response curve for glucagon is

Fig. 3. Effect of dehydroascorbate on the rate of ferricyanide reduction by perfused rat liver

Livers were perfused as described in the Materials and methods section. The ferricyanide concentration was 0.5 mM. Other details were as given in Table 1. Dehydroascorbate at 1 mM (○) or 0.1 mM (△) (final concentration) was added after 16 min of perfusion (arrow). The release of ferricyanide-reducing substances formed from dehydroascorbate by the liver was assessed by adding perfusate samples from a perfusion containing only 1 mM-dehydroascorbate to tubes with 0.5 mM-ferricyanide. The A_{420} was read after incubating the tubes for 10 min at 37°C (●). Representative data are shown.

shown. Glucagon (1 μ M) inhibited the rate of ferricyanide reduction from 0.21 to 0.15 μ mol/min per g liver. Half-maximal inhibition occurred at approx. 0.1 nM-glucagon. In Fig. 4(b) the dose-response curve for glucagon on the rate of glucose release is shown; half-maximal stimulation also occurred at approx. 0.1 nM-glucagon. These findings indicate a close correlation between the effects of glucagon on the events occurring both at the plasma membrane and within the cell. In Fig. 5 the time course for the effect of 10 nM-glucagon is shown. The inhibitory effect appeared to occur within minutes following addition of glucagon to the perfusate.

To assess the possible role of cyclic AMP in the glucagon-mediated inhibition of ferricyanide reduction (Fig. 4), the effects of various concentrations of the cyclic nucleotide on the rates of ferricyanide reduction and glucose release were examined. Fig. 6 shows that whereas cyclic AMP markedly stimulated the rate of glucose release (approx. 5-fold), there was no significant effect on the rate of ferricyanide reduction. Dibutyl cyclic AMP at the

Fig. 4. Effect of glucagon on the rate of ferricyanide reduction and glucose release by perfused rat liver

Livers were perfused as described in the Materials and methods section. The ferricyanide concentration was 0.5 mM. Perfusions were conducted in a recirculating mode for a total of 40 min. For ferricyanide reduction (a), the initial rate (0–16 min) was determined. Glucagon was added at 16 min and the final rate (18–38 min) was determined. The linear final rates are shown. For glucose release (b), 50 μl of the perfusate was taken for glucose determination at 4, 16, 20 and 38 min (Werner *et al.*, 1970). The amount of glucose released between 16 and 38 min is shown. Each point represents a single perfusion.

Fig. 5. Time course for the effect of glucagon on the rate of ferricyanide reduction by perfused rat liver

Details were as given for Fig. 4 or as given in the Materials and methods section. Glucagon (10 nM) was added at 18 min (arrow). Data from one representative experiment of a total of three liver perfusions are shown.

maximal dose of 0.1 mM also had no significant effect on the rate of ferricyanide reduction (Fig. 6). These findings support the view that the effects of glucagon are direct and do not involve an intermediary step of membrane protein phosphorylation.

As reported previously, ferrocyanide oxidation by the perfused liver was negligible (Clark *et al.*, 1981). Nevertheless the possibility remained that the glucagon-mediated inhibition of ferricyanide reduction was the result of a pronounced acceleration in the rate of ferrocyanide oxidation. Fig. 7 shows that the rate of ferrocyanide oxidation was unaffected by 1 μM -glucagon and continued at a linear rate equivalent to approx. 5% of the rate of ferricyanide reduction.

In general the data of Fig. 4 were unexpected since, as indicated above, studies with isolated purified plasma membranes had indicated that glucagon was stimulatory. Several possibilities

Fig. 6. *Effect of cyclic AMP and dibutyryl cyclic AMP on the rate of ferricyanide reduction and glucose release by perfused rat liver*

Details were as given for Fig. 4 or as given in the Materials and methods section. Cyclic AMP (Δ , $\blacktriangle$) or dibutyryl cyclic AMP ($\square$) was added at 16 min and the rates of ferricyanide reduction (open symbols) and glucose release (closed symbols) were determined over the next 22 min. Where appropriate means $\pm$ S.E.M. have been calculated and the number of perfusions is shown in parentheses.

emerge to explain this difference. For the intact organ studies, the vectorial relationships between the unknown intracellular redox donor and the extracellular oxidant, ferricyanide, are defined. Glucagon may directly affect ferricyanide binding, ferricyanide reduction or modify the enzyme(s) involved in the trans-plasma membrane reduction process. Glucagon may indirectly affect the rate of ferricyanide reduction by altering the catalytic activity of enzymes concerned with the production of the intracellular reductant. In contrast with the intact organ, where the reduction of ferricyanide is transmembraneous, the isolated plasma membrane preparation is heterogeneous with no structured vectorial relationships. Reduction of ferricyanide may be occurring on the inside surface, the outside surface or from inside to outside or from outside to

Fig. 7. *Effect of glucagon on the rate of ferrocyanide oxidation by perfused rat liver*

Livers were perfused as described in the Materials and methods section except that sodium ferrocyanide (0.5 mM) replaced the ferricyanide. After the washout period (5 min) a recirculating perfusion was conducted for a total of 40 min. The initial rate (0–16 min) of ferricyanide reduction was determined. Glucagon ($1\mu\text{M}$) was added at 16 min (arrow) and the final rate (18–38 min) was determined. The change in concentration of ferricyanide was determined by monitoring the increase in A_{420} of the perfusate.

inside. Since the membranes are mostly flat, open sheets, all four reactions are possible and any hormonal effect involving presumably the extracellular receptors is difficult to interpret.

The present findings suggest a relationship between trans-plasma membrane redox activity and the early events in glucagon action (i.e. receptor–adenylate cyclase relationships). It remains to be investigated whether ferricyanide has any effects on glucagon-mediated activation of adenylate cyclase in liver.

In summary, the present findings indicate that the reduction of ferricyanide by perfused liver is largely unaffected by energy status of the cells and operates independently of the state of reduction of either the cytosolic or mitochondrial $[\text{free NADH}]/[\text{free NAD}^+]$ ratios. It is stimulated in an unknown manner by dihydroxyacetone and by dehydroascorbate and it is regulated by glucagon (inhibitory).

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