J. Clin. Chem. Clin. Biochem.
Vol. 22, 1984, pp. 705–509

Rat Liver 3-Oxo-5 α -steroid Δ^4 -Dehydrogenase. Modulation of Enzyme Activity by Changes in Phosphorylation State

By S. W. Golf and V. Graef

Zentrum für Klinische Chemie und Pathobiochemie Klinikum der Justus-Liebig-Universität Gießen

(Received May 14/August 24, 1984)

In honour of Prof. Dr. Ladislaus Róka on the occasion of his sixtyfifth birthday

Summary: 3-Oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase ("NADH-5 α -reductase", EC 1.3.1.?) is rapidly inactivated in the presence of 17 β -hydroxy-4-androsten-3-one (testosterone). This inactivation is prevented by increasing the phosphate concentration. When the enzyme assay is carried out in Tris-HCl, only a small activity (1.7 nmol $\times$ min⁻¹ $\times$ mg⁻¹) is observed which may be further decreased by addition of phosphatases. Addition of the phosphatase inhibitor dextran sulphate or ATP, Mg⁺⁺ and c-AMP results in a significant increase of activity (228% and 273%, respectively) compared with the Tris-HCl control. Glycerol 2-phosphate and glycerol 3-phosphate have a stabilizing effect on 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase by decreasing the K_m towards the substrate testosterone from 1.2×10^{-5} mol/l to 3.3×10^{-6} mol/l. V remains unchanged. Half maximal velocity (testosterone reduction) is achieved with 20 μ mol/l glycerol 2-phosphate and glycerol 3-phosphate. Addition of c-AMP dependent protein kinase (EC 2.7.1.37) to a microsomal preparation pretreated with alkaline phosphatase (EC 3.1.3.1) results in a significant increase of 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase activity compared with the control.

Modulation der Enzymaktivität der 3-Oxo-5 α -steroid Δ^4 -Dehydrogenase aus der Rattenleber durch Veränderung des Phosphorylierungszustandes

Zusammenfassung: Die 3-Oxo-5 α -steroid: NAD⁺ Δ^4 -Oxidoreduktase („NADH-5 α -Reduktase“, EC 1.3.1.?) wird in Gegenwart von 17 β -Hydroxy-4-androsten-3-on (Testosteron) rasch inaktiviert. Steigende Phosphat-Konzentrationen verhindern zunehmend diese Inaktivierung. Bei einer Durchführung des enzymatischen Ansatzes in Tris-HCl-Puffer ist nur eine geringe Enzymaktivität (1,7 nmol $\times$ min⁻¹ $\times$ mg⁻¹) meßbar, wobei die Aktivität durch Zusatz von Phosphatase weiter vermindert werden kann. Die Gegenwart des Phosphatase-Inhibitors Dextransulfat und von ATP, Magnesium und c-AMP verursacht im Vergleich mit der Tris-HCl-Kontrolle einen signifikanten Anstieg (228% und 273%) der Enzymaktivität. Glycerin-2- und Glycerin-3-phosphat haben eine stabilisierende Wirkung auf die 3-Oxo-5 α -steroid: NAD⁺ Δ^4 -Oxidoreduktase, indem die K_m für Testosteron von $1,2 \times 10^{-5}$ mol/l auf $3,3 \times 10^{-6}$ mol/l vermindert wird. V bleibt unverändert. Die halb-maximale Reaktionsgeschwindigkeit (Reduktion von Testosteron) wird bei ca. 20 μ mol/l Glycerin-2- und Glycerin-3-phosphat erreicht. Eine mit alkalischer Phosphatase (EC 3.1.3.1) vorbehandelte Mikrosomenpräparation weist nach Zugabe einer c-AMP abhängigen Proteinkinase (EC 2.7.1.37) einen im Vergleich zur Kontrolle signifikant erhöhten Anstieg der 3-Oxo-5 α -steroid: NAD⁺ Δ^4 -Oxidoreduktase-Aktivität auf.

Introduction

3-Oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase from female rat liver microsomes undergoes a diurnal variation of activity which is related to phosphatases

present in microsomes (1) and possibly to the testosterone concentration in rat plasma (2). A potential, yet unexplored mechanism for this circadian modulation of 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreduc-

tase activity is the reversible change in phosphorylation state. A large body of evidence has suggested that other microsomal enzymes such as the cholesterol-7 α -hydroxylase (EC 1.14.13.17) (3) and the hydroxymethylglutaryl CoA reductase (EC 1.1.1.34) (4), which in part have a diurnal variation of activity, also are regulated by phosphatases and protein kinases.

3-Oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase in the endoplasmatic reticulum of the rat liver cell together with 4,5 α -dihydrocortisone: NADP⁺ Δ^4 -oxidoreductase ("NADPH-5 α -reductase", EC 1.3.1.4) converts 4-en-3-oxosteroids to 5 α -reduced products (5). These 5 α -dihydrosteroids are the biological active compounds, such as 17 β -hydroxy-5 α -androstane-3-one (5 α -dihydrotestosterone) in the prostate (6) or 5 α -pregnane-3,20-dione (5 α -dihydroprogesterone) in the hypothalamus (7). In the liver cell of the female rat 11 β ,17 α ,21-trihydroxy-5 α -pregnane-3,20-dione (5 α -dihydrocortisol) and 11 β ,21-dihydroxy-5 α -pregnane-3,20-dione (5 α -dihydrocorticosterone) cause a significant decrease of enzyme activities that limit the gluconeogenesis rate, such as pyruvate carboxylase (EC 6.4.1.1) or phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32), accompanied by a concomitant decrease in insulin concentration (8). Nothing is known about the influence of other 5 α -dihydrosteroids on biochemical pathways in the liver, but it must be assumed that the effect of steroids on enzymatic reactions in the liver cell may be regulated by 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase.

Materials and Methods

Reagents

NADH, ATP, c-AMP and acid phosphatase (potato) were purchased from Boehringer, D-6800 Mannheim. Steroids, buffer reagents, magnesium chloride and sheep anti-alkaline phosphatase antibodies were obtained from Merck, D-6100 Darmstadt. Glycerol 3-phosphate and glycerol 2-phosphate, alkaline phosphatase (*E. coli*) and c-AMP dependent protein kinase (rabbit muscle) were purchased from Sigma, D-8000 München.

Animals

Female Wistar rats (Zentralinstitut für Versuchstiere, D-3000 Hannover) received water and feed ad libitum and were exposed to a light-dark cycle with light from 6.00 to 18.00. They were sacrificed at 8.00 and liver microsomes were prepared immediately (9). The protein concentration was determined by the biuret method (9).

3-Oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase assay

The reaction mixture (3.0 ml) contained 0.2 mol/l potassium phosphate buffer pH 6.5, 300 nmol testosterone, 2.4 μ mol NADH and 0.08–0.74 mg microsomal protein. After 10 minutes the reaction was stopped by ether extraction and the steroid substrate and products were determined by gas chromatography following CrO₃ oxidation of steroids (10).

Effect of protein kinase on 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase

Microsomal protein (0.13 mg) was incubated (15 min) in the presence of 0.2 mol/l potassium phosphate buffer pH 6.5, 0.41 units alkaline phosphatase, 0.132 mmol/l ATP, 6.4 μ mol/l c-AMP, 1.11 mmol/l MgCl₂, 62 mmol/l glycerol 2-phosphate or glycerol 3-phosphate, assay volume 1.0 ml. After phosphatase pretreatment 4 units of anti-alkaline phosphatase antibodies (sheep) and 80 picomol units c-AMP dependent protein kinase were added; the control contained no protein kinase. After incubations of 15 and 30 min, 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase activity was measured by addition of 0.8 μ mol NADH and 100 nmol testosterone as described above.

The results described represent the mean values obtained from at least four repetitions of the experiments. The probability level chosen for significant differences was $P < 0.05$ (t-test).

Results and Discussion

3-Oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase from rat liver microsomes is an enzyme which can be differentiated from the NADPH dependent 5 α -reductase (EC 1.3.1.4) by inhibitors (5), solubilization with detergents (11), affinity labeling with nucleoside analogues (10), diurnal variation of activity (1, 2, 12) and administration of chlorinated hydrocarbons to rats (13). 3-Oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase is phosphate or arsenate dependent (14). The activity of 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase rapidly declines when the enzyme assay is carried out in low phosphate concentration (0.05 mol/l) (fig. 1). The enzyme has an unusually short half-life (2.5 min) under these conditions in presence of testosterone. The activity may be stabilized by a higher phosphate concentration (fig. 1).

Fig. 1. Specific activity of 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase as a function of time of incubation in potassium phosphate buffer pH 6.5. $\square$ — $\square$ 0.5 mol/l; $\circ$ — $\circ$ 0.05 mol/l; $\blacksquare$ — $\blacksquare$ 0.5 mol/l in the presence of 62 mmol/l glycerol 3-phosphate $\bullet$ — $\bullet$ 0.05 mol/l in the presence of 62 mmol/l glycerol 2-phosphate.

The exact nature of the phosphate ion effect is not known, but it may be presumed that it acts as an inhibitor of phosphatases, which, in turn modulate 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase activity. Acid and alkaline phosphatases are present in the liver cell (15) and in microsomes (16). In addition, it was shown that testosterone pretreatment of microsomes greatly enhances acid phosphatase activity (EC 3.1.3.2) (1). When the enzyme assay is carried out in the presence of the phosphatase inhibitor, dextran sulphate, and antibodies (sheep) against alkaline phosphatase, both partially prevent the decrease of 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase activity (tab. 1).

Glycerol 2-phosphate and glycerol 3-phosphate in the enzyme assay also prevent the rapid inactivation of 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase. In low phosphate concentration (0.05 mol/l) the half-life of the enzyme is extended from 2.5 minutes to 6 minutes (fig. 1). A distinct effect of glycerol 2-phosphate and glycerol 3-phosphate on 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase is also seen in 0.2 mol/l phosphate buffer (fig. 2), a clear indication that its effect is not a phosphate ion effect. The K_m of 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase for the substrate testosterone is decreased from 1.2×10^{-5} mol/l to 3.3×10^{-6} mol/l while the maximal testosterone reduction remains unchanged. Both glycerol 2-phosphate and glycerol 3-phosphate have the same effect (fig. 2).

Tab. 1. 3-Oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase activity of rat liver microsomes in Tris-HCl buffer in the presence of several modifiers.

The incubation was carried out in 0.2 mol/l Tris-HCl buffer pH 6.5 (3.0 ml), containing 0.74 mg microsomal protein, 3.0 μ mol NADH, 300 nmol testosterone, 3 μ mol ATP and Mg⁺⁺, 300 nmol c-AMP, 4 units anti-alkaline phosphatase antibodies (sheep), 10 units alkaline phosphatase from calf intestines and 40 units acid phosphatase from potato as indicated. Incubation was for 10 minutes at 37°C.

Effectors present in incubation mixture	Specific activity nmol testosterone reduced per min and mg		
	$\bar{x} \pm SD$	%	Significance
No (control)	1.68 $\pm$ 0.15	100	
Dextran sulphate (10 mg)	3.83 $\pm$ 0.36	228	P < 0.05
ATP, c-AMP, Mg ⁺⁺	4.59 $\pm$ 0.46	273	P < 0.05
Phosphatase antibodies	1.98 $\pm$ 0.22	118	P < 0.10
Acid phosphatase	1.65 $\pm$ 0.16	83	P < 0.05
Alkaline phosphatase	0.36 $\pm$ 0.05	21	P < 0.05

Glycerol 3-phosphate, for example, is present in the rat liver cell at a concentration of 200–500 μ mol/l (17). This is sufficient for an in vivo modulation of 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase activity as is seen in figure 2. Half maximal 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase activity is obtained with 20 μ mol/l glycerol 3-phosphate.

Fig. 2. Effect of glycerol phosphate on 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase, *Lineweaver-Burk* plot

- a) varying testosterone concentration; —●— control, $b = 8.02 \times 10^{-4}a + 6.7 \times 10^{-2}$, corr. = 0.925, $V = 14.91$ nmol $\times$ min⁻¹ $\times$ mg⁻¹, $K_m = 1.2 \times 10^{-2}$ mmol/l; —○— glycerol 3-phosphate (62 mmol/l), $b = 3.27 \times 10^{-4}a + 9.85 \times 10^{-2}$, corr. = 0.981, $V = 10.15$ nmol $\times$ min⁻¹, $K_m = 3.32 \times 10^{-3}$ mmol/l; —×— glycerol 2-phosphate, $b = 2.43 \times 10^{-4}a + 7.32 \times 10^{-2}$, corr. = 0.993, $V = 13.65$ nmol $\times$ min⁻¹, $K_m = 3.31 \times 10^{-3}$ mmol/l.
- b) varying glycerol 3-phosphate concentration; $b = 1.44 \times 10^{-3}a + 6.98 \times 10^{-2}$, corr. = 1.0, $V = 14.32$ nmol $\times$ min⁻¹ mg⁻¹, $K_m = 2.06 \times 10^{-2}$ mmol/l.

There are several possible explanations for the observed effects of glycerol 2- and glycerol 3-phosphate on 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase. The liver cytosol contains a NAD⁺/NADH linked glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) which catalyses the reversible oxidation of dihydroxyacetone phosphate and glycerol 3-phosphate. As a result, high glycerol 3-phosphate concentration in the enzyme assay could lead to continuously reduced NADH when this coenzyme is oxidized during the 5 α -reduction, accompanied by a possibly higher 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase activity. This assumption, however, is not probable, since glycerol 2-phosphate has the same effect on 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase as glycerol 3-phosphate. In addition the glycerol 3-phosphate dehydrogenase thermodynamically favours the formation of NAD⁺ and glycerol 3-phosphate (18). Secondly, an increased 5 α -reduction of testosterone in the presence of glycerol 3-phosphate or glycerol 2-phosphate might be possible via the NADPH-cytochrome reductase (EC 1.6.2.4). This enzyme is involved in the 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase enzyme system (19) and normally is able to oxidize NADH (substrate cytochrome c) to an extent of less than 1 nmol $\times$ min⁻¹ $\times$ mg⁻¹ (20). In the presence of glycerol 3-phosphate, however, no activation of NADH oxidation by purified NADPH-cytochrome reductase was observed (20). In addition, the change of K_m of 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase for the substrate testosterone indicates a direct modulation by glycerol 2-phosphate and glycerol 3-phosphate of the 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase protein conformation (fig. 2).

Glycerol 2-phosphate sometimes is used as an activator of protein kinases (21). The involvement of protein kinases in the increase of 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase activity by glycerol 2-phosphate and glycerol 3-phosphate is indeed possible as seen in table 1. Addition of ATP, Mg⁺⁺ and c-AMP to the 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase assay causes a significant 173% increase of activity compared with the control. If this change of activity is caused by a protein kinase, then this enzyme must be present in microsomes. All protein kinases from liver cells described so far have been located in the cytosol, although in other organs, such as the brain, almost 60% of c-AMP dependent protein kinases is associated with the particulate subcellular fraction (22). The observed effects could be caused by a displaced cytoplasmic protein kinase or by an enzyme which is an integral component of the microsomal membrane.

A direct phosphorylation of microsomal 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase is not proved by these experiments. This only will be possible with a purified enzyme, but such a preparation is not available (11).

Modification of the above experiments, however, clearly indicates that 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase activity is being modulated by phosphorylation and dephosphorylation reactions (fig. 3). Microsomes are incubated with an alkaline phosphatase so that the 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase activity rapidly declines (1). Then alkaline phosphatase antibodies, c-AMP dependent protein kinase and its activators (ATP, c-AMP, Mg⁺⁺) are added while the control contains no protein kinase. It is seen in figure 3 that at least part of the 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase activity which was lost by a phosphatase hydrolysis can be restored by a c-AMP dependent protein kinase catalysed reaction.

Fig. 3. Effects of protein kinase on 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase activity. Microsomes were incubated for 15 min in the presence of ATP, c-AMP, MgCl₂, alkaline phosphatase and glycerol phosphate. Anti-alkaline phosphatase antibodies and c-AMP dependent protein kinase were then added. At the indicated time corresponding samples were taken from the incubation mixture to determine 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase activity after addition of NADH and testosterone. —x— glycerol 3-phosphate, —o— glycerol 2-phosphate.

Our results offer considerable proof that the substantial alteration of microsomal 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase activity is caused by a phosphorylation-dephosphorylation reaction. These results constitute the first evidence that the formation of active androgens in the female rat liver is subject to a regulation as expressed by the state of phosphorylation of 3-oxo-5 α -steroid: NAD⁺ Δ^4 -oxidoreductase.

References

1. Golf, S. W. & Graef, V. (1984) Hoppe-Seylers Z. Physiol. Chem. 365, 1271–1276.
2. Graef, V. & Golf, S. W. (1979) J. Steroid Biochem. 11, 1299–1302.
3. Goodwin, C. D., Cooper, B. W. & Margolis, S. (1982) J. Biol. Chem. 257, 4469–4472.
4. Hunter, C. F. & Rodwell, V. W. (1980) J. Lipid Res. 21, 399–405.
5. Graef, V. & Golf, S. W. (1975) Z. Klin. Chem. Klin. Biochem. 13, 333–339.
6. Lesser, B. & Bruchowsky, N. (1973) Biochim. Biophys. Acta 308, 426–437.
7. Nowak, F. V. & Karavolas, H. J. (1974) Endocrinology 94, 994–997.
8. Golf, S. W., Graef, V. & Bepperling, F. (1984) Steroids, in press.
9. Golf, S. W., Graef, V. & Staudinger, H. (1980) Hoppe-Seylers Z. Physiol. Chem. 355, 1063–1069.
10. Golf, S. W. & Graef, V. (1982) Steroids 40, 1–9.
11. Graef, V., Golf, S. W. & Rempeters, G. (1978) J. Steroid Biochem. 9, 369–371.
12. Golf, S. W. & Graef, V. (1979) Hoppe-Seylers Z. Physiol. Chem. 360, 269.
13. Graef, V., Golf, S. W. & Goerz, G. (1979) Arch. Toxicol. 43, 115–120.
14. Leybold, K. & Staudinger, H. (1963) Biochem. Z. 337, 320–327.
15. Di Pietro, D. L. & Zengerle, S. (1967) J. Biol. Chem. 242, 3391–3396.
16. Hoffmann, L. M. & Di Pietro, D. L. (1977) Am. J. Obstet. Gynecol. 114, 1087–1096.
17. Liu, E. C. C. (1977) Ann. Rev. Biochem. 46, 782.
18. Lehninger, A. L. (1951) J. Biol. Chem. 190, 345–349.
19. Golf, S. W. & Graef, V. (1978) J. Steroid Biochem. 9, 1087–1092.
20. Vogel, E. (1984) Zentr. für Biochem., University Gießen, personal communication.
21. Cogeli, J. M. & Dobson, J. G. (1981) Anal. Biochem. 110, 331–337.
22. Maeno, H., Johnson, E. H. & Greengard, P. (1971) J. Biol. Chem. 246, 134–142.

Dr. Sighart Golf
Zentrum für Klinische Chemie
und Pathobiochemie
an den Kliniken
der Justus-Liebig-Universität
Friedrichstraße 24
D-6300 Gießen