PARTIAL PURIFICATION OF A 10-FORMYL-TETRAHYDROFOLATE: NADP OXIDOREDUCTASE FROM MAMMALIAN LIVER

C. Kutzbach and E. L. R. Stokstad

Department of Nutritional Sciences
University of California
Berkeley, California, USA

Received November 21, 1967

Osborn *et al.* (1957) reported an enzymatic deacylation of 10-CHO-H_4PteGlu* according to equation 1 by a preparation from beef liver

$$10\text{-CHO-}H_4\text{PteGlu} + H_2O \longrightarrow H_4\text{PteGlu} + HCOOH \qquad (1)$$

acetone powder. Full activity required catalytic amounts of either NADP or NADPH. We became interested in the possible involvement of this enzyme in the regulation of the tissue levels of free H_4PteGlu. Our work led to the finding of a different, oxidative deformylation of 10-CHO-H_4PteGlu according to equation 2.

$$10\text{-CHO-}H_4\text{PteGlu} + NADP^+ + H_2O \longrightarrow H_4\text{PteGlu} + CO_2 + NADPH + H^+ \qquad (2)$$

This communication describes the partial purification of this new enzyme activity from pig liver and the evidence for reaction 2.

Materials and Methods. A gift of ± 5-CHO-H_4PteGlu was received from the American Cyanamid Corp., Lederle Laboratories Division. The 10-CHO-H_4PteGlu was made from the 5-isomer as described by Brode and Jaenicke (1961), and H_4PteGlu according to Ramasastri and Blakley (1962). Crystalline formylase (formate:tetrahydrofolate ligase E.C.6.3.4.3.) from *C. cylindrosporum* or *C. acidi urici* was a gift of Dr. J. Rabinowitz. All optical measurements were made in a Gilford 2000 spectrophotometer with automatic changer in cuvettes with 1 cm light path. Protein was

Abbreviations: GDH = glutamate dehydrogenase; H_4PteGlu = tetrahydrofolic acid; KG = ketoglutarate; 2-me = 2-mercaptoethanol.

determined from the absorption at 280 and 260 mμ (Layne, 1957).

Enzyme activity was determined from the initial rate of increase in O.D. at 300 mμ due to the formation of H_4PteGlu. The cuvettes contained in a total volume of 2 ml: 100 μmoles Tris buffer pH 7.7, 200 μmoles 2-me, 0.2 μmoles ± 10-CHO-H_4PteGlu and enzyme; 0.2 μmoles NADP were added to start the reaction. A small contribution from the formation of NADPH can be avoided by adding excess GDH, KG and NH_4^+. The molar extinction coefficient for the conversion 10-CHO-H_4PteGlu ⟶ H_4PteGlu at 300 mμ was determined as 21 · 10^3 cm^2 · mol^{-1} using the formylase reaction (equation 3): the decrease at 300 mμ was compared to the amount of 10-CHO-H_4PteGlu formed as determined from the extinction at 355 mμ after acidification. Formate was assayed enzymatically with formylase (Rabinowitz and Pricer, 1962), after removal of remaining 10-CHO-H_4PteGlu with Norite.

Purification of the Enzyme

Table 1. Purification of 10-CHO-H_4PteGlu:NADP Oxidoreductase from Pig Liver

Fraction	Volume ml	Protein mg/ml	Activity $U^*.mg^{-1}.10^3$	Activity Total Units
pH 6 supernatant	1700	17.0	1.5	42
AS 30-45%	450	34.0	2.3	36
DEAE-Cellulose	500	7.0	5.5	19
DEAE-Sephadex	170	1.5	44.5	11
AS 0-80%	3	31.5	70	7
Hydroxylapatite	4	2.5	305	3 (4.5)**

*One unit is equal to 1 μmole x $min.^{-1}$.

**Calculated yield if total AS 0-80 fraction had been used for the last step.

A scheme by which an overall 200-fold purification has been achieved is shown in Table 1. Small samples of the first fractions were subjected to dialysis and ultracentrifugation to obtain a clear supernatant for the spectrophotometric assay.

Frozen pig liver was homogenized with 3 vol. of standard buffer (0.05 M phosphate pH 7.2, 0.001 M 2-me), the homogenate adjusted to

pH 6.0 with 4 M acetic acid and centrifuged 1 hour at 15,000 g. The supernatant was fractionated with solid $(NH_4)_2SO_4$ and most of the activity recovered in the 30-45% fraction. This fraction was dialyzed overnight against standard buffer and applied to a 9.5 x 14 cm column of DEAE-Cellulose (Whatman DE 22). After washing with 500 ml buffer, the activity was eluted with 0.15 M KCl in buffer. This fraction was applied on a 4.5 x 16 cm column of DEAE-Sephadex A 50 and eluted with a linear gradient formed from 500 ml each 0.15 M and 0.5 M KCl in buffer. Active fractions (around the middle of the gradient) were pooled and solid $(NH_4)_2SO_4$ added to 80% saturation. After centrifugation for 30 min. at 30,000 x g, the precipitate was suspended in 7 ml buffer. The suspension was centrifuged for 1 hour at 30,000 x g and all the activity found in the precipitate. This was dissolved in 3 ml buffer. Two ml of this fraction were further purified on hydroxylapatite (Levin, 1962). After 6 hours dialysis, the sample was applied to a 1.2 x 2.5 cm column and the column washed with 20 ml buffer. The enzyme was eluted with 0.07 M phosphate buffer. The enzyme activity has also been demonstrated in homogenates from rat liver and extracts from both beef and pig liver acetone powder.

Properties. The purified enzyme has a specific requirement for NADP. NADPH is not effective; NAD gives less than 5% of the full activity. No reaction is observed with 5-CHO-H_4PteGlu. The pH-optimum is between pH 7.5 and 8.5 in both phosphate and TRIS buffer, but the reaction is approximately 30% faster in TRIS buffer.

Reaction Products. The formation of NADPH is indicated by the increase in O.D. at 340 mμ and the decrease after addition of GDH, KG and NH_4^+. Formation of NADPH is not observed when 10-CHO-H_4PteGlu is replaced by formate and/or H_4PteGlu. The formation of CO_2 was demonstrated both by standard Warburg manometry and as $^{14}CO_2$ (Table 2) when observed in a system in which 10-^{14}CHO-H_4PteGlu was formed *in situ* by the formylase reaction (equation 3).

$$H_4PteGlu + H^{14}COOH + ATP \longrightarrow 10\text{-}^{14}CHO\text{-}H_4PteGlu + ADP + Pi \quad (3)$$

$$10\text{-}^{14}CHO\text{-}H_4PteGlu + NADP^+ + H_2O \longrightarrow H_4PteGlu + {}^{14}CO_2 + NADPH + H^+ \quad (2)$$

$$H^{14}COOH + ATP + NADP^+ + H_2O \longrightarrow {}^{14}CO_2 + NADPH + ADP + Pi + H^+ \quad (4)$$

Table 2. Formation of $^{14}CO_2$ from ^{14}C-HCOOH

	System	$^{14}CO_2$	
		cpm	μmoles
Exp. 1	Complete	78,144	1.34
	NADP omitted	7,866	0.14
	10-CHO-H_4PteGlu omitted	5,492	0.09
	10-CHO-H_4PteGlu replaced by H_4PteGlu	66,818	1.14
Exp. 2	Complete	54,480	0.94
	ATP omitted	3,032	0.05
	Formylase omitted	1,346	0.02
	Enzyme omitted	1,386	0.02

The incubations were performed in Warburg vessels under a H_2 atmosphere for 1 hr. at 30°C. The vessels contained: in the main compartment, 200 μmoles TRIS pH 7.7, 100 μmoles 2-me, 0.25 μmoles ± 10-CHO-H_4PteGlu or 0.5 μmoles ± H_4PteGlu as indicated, 5 μmoles ATP, 5 μmoles ^{14}C-HCOOH (3.6×10^5 dpm/μmole), 50 μmoles $MgCl_2$, 800 units of formylase and 3 mg enzyme (spec. act. 7.6) in a total volume of 1.1 ml; sidearm 1: 0.15 ml 2×10^{-3} M NADP; sidearm 2: 0.1 ml 2.5 M $HClO_4$; center well: a piece of filter paper soaked with 0.15 ml 1 N NaOH. The reaction was started by introducing the NADP from sidearm 1. After it had been terminated by addition of the acid from sidearm 2, the vessels were shaken for 10 min. for absorption of CO_2. The contents of the center well were then transferred with several washings to a graduated test tube and filled to 2.0 ml. An 0.5 ml aliquot was counted in a Beckman CPM 100 liquid scintillation counter in 10 ml of Bray solution (Bray, 1960).

The results of Table 2 also indicate H_4PteGlu as the other product along with CO_2 since a catalytic amount of either 10-formyl- or free H_4PteGlu is sufficient to oxidize formate to CO_2 according to equation 4. Very little oxidation of formate to CO_2 was observed in the absence of either the folate compound or the complete formylase system.

If the formylase system is added to the spectrophotometric assay, the increase at 300 mμ is markedly diminished because the product H_4PteGlu is immediately converted back to the substrate. In contrast, the extinction at 340 mμ increases more rapidly and at an almost constant rate until one

substrate becomes limiting (Fig. 1). We believe that the rapid decrease of the reaction rate which we always observe in the standard assay is due to inhibition by H_4PteGlu formed in the reaction. The addition of the formylase system releases this inhibition, which had also been mentioned by Osborn et al. and may have regulatory significance.

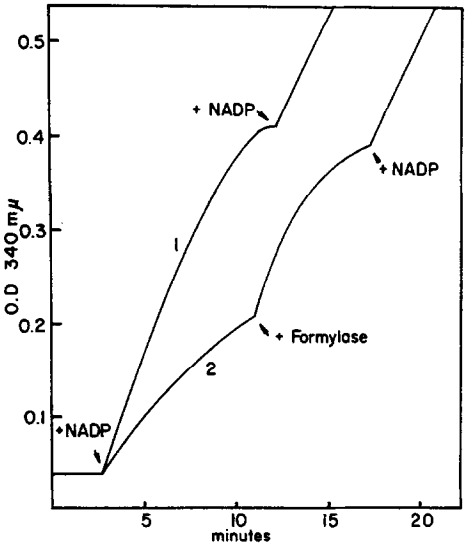

Fig.1: Effect of the formylase system on the formation of NADPH.

The cuvettes contained in addition to the standard assay system: 20 μmoles formate, 50 μmoles Mg Cl_2, 2.5 μmoles ATP and 800 units formylase from start (1) or added later (2).

Reaction Stoichiometry. The stoichiometry of the reaction was investigated in two ways: (1) the formation of H_4PteGlu and NADPH and the disappearance of 10-CHO-H_4PteGlu were found by optical assay to be of the same magnitude (Table 3); (2) the radioactive assay system coupled with formylase established a 1:1 relationship between limiting amounts of NADP and the formation of $^{14}CO_2$ (Table 4). Both results are in accordance with equation 2.

Formation of formate could not be detected when purified enzyme was incubated with 10-CHO-H_4PteGlu and NADP. However, several enzyme samples

Table 3. Stoichiometric formation of NADPH and H_4PteGlu from 10-CHO-H_4PteGlu

	NADPH		H_4PteGlu		10-CHO-H_4PteGlu	
Exp	ΔE_{340}	mμmoles	ΔE_{300}	mμmoles	ΔE_{355}	mμmoles
1	0.19	+62	0.61	+58	0.89	-71
2	0.20	+65	0.80	+76	0.97	-77
3	0.24	+78	0.72	+69	0.87	-70
4	0.16	+52	0.53	+51	0.75	-60

Reaction mixtures were as in the standard assay except for alterations in the amounts of 10-CHO-H_4PteGlu and NADP. In Exp. 1-3, 10-CHO-H_4PteGlu was the limiting substrate and 100 or 200 (Exp. 2) mμmoles NADP were present. In Exp. 4 NADP was limiting and approx. 100 mμmoles of the folate in the active diastereomeric form were present. Enzyme was 0.02 ml of the AS 0-80 fraction in Exp. 1 and 0.05 ml of the hydroxylapatite fraction in the others. A blank without enzyme was run at the same time. The reaction was followed at 300 mμ until there was no further change in O.D. 10 μmoles of each KG and NH_4^+ were then added bringing the volume to 2.0 ml, and the decrease at 340 mμ upon the addition of GDH recorded. Following this, ΔE_{300} between the sample and the blank was measured and corrected for the difference at zero time. Immediately thereafter, 1 drop of 2.5 N $HClO_4$ was added to each cuvette and the increase at 355 mμ measured after 10 min., if necessary, after centrifugation of the samples.

Table 4. Stoichiometric formation of $^{14}CO_2$ with a limiting amount of NADP

NADP (μmoles)	$^{14}CO_2$ formed (μmoles)
0.13	0.13
0.39	0.33

Conditions were as in Table 2 except that 1 μmole of ± H_4PteGlu and smaller amounts of NADP as indicated were used. NADP was placed in the main compartment and the enzyme in sidearm 1. Enzyme was 0.05 ml of the AS 0-80 fraction.

after frozen storage for some weeks showed about 30% of full activity when measured as deformylation of 10-CHO-H_4PteGlu in the absence of NADP, and an equivalent amount of formate was found under these conditions. This suggests that a labile formyl compound is an intermediate in the

reaction and may undergo nonenzymatic cleavage instead of oxidation to CO_2 in the absence of NADP under certain conditions.

A greatly reduced oxidation of formate to CO_2 has been observed in folate-deficient animals and tissues (Plaut et al., 1950; Friedman et al., 1954). It is widely believed that the oxidation of formate to CO_2 is catalyzed in vivo by catalase, and it has been shown that this enzyme is less active in folate deficiency (Friedman et al., 1954). The demonstration of a 10-CHO-H_4PteGlu: NADP oxidoreductase offers a pathway which directly involves H_4PteGlu in the oxidation of formate to CO_2 and provides an alternate explanation.

Acknowledgment. This work was supported in part by a grant from the National Institutes of Health, No. AM 08171.

REFERENCES

Bray, G.A., Anal. Biochem. 1, 279 (1960).
Brode, E., and Jaenicke, L., Liebigs Ann. Chem. 647, 174 (1961).
Friedman, B., Nakada, H.I., and Weinhouse, S., J. Biol. Chem. 210, 413 (1954).
Layne, E., Methods in Enzymology III, 447 (1957).
Levin, O., Methods in Enzymology V, 27 (1962).
Osborn, M.J., Hatefi, Y., Kay, L.D., and Huennekens, F.M., Biochem. Biophys. Acta 26, 208 (1957).
Plaut, G.W.E., Betheil, J.J., and Lardy, H.A., J. Biol. Chem. 184, 795 (1950).
Rabinowitz, J.C., and Pricer, Jr., W.E. in H.U. Bergmeyer: Methoden der enzymatischen Analyse, Verlag Chemie 1962, p. 308.
Ramasastri, B.V., and Blakley, R.L., J. Biol. Chem. 237, 1982 (1962).