BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **226,** 187–192 (1996)
ARTICLE NO. 1331

Reduction of Ferricyanide by Thiamine or Thiamine Pyrophosphate[1]

Guoyu Li, Mark E. Cook, and Weidong Wu[2]

Department of Poultry Science, University of Wisconsin, Madison, Wisconsin 53706

Received July 24, 1996

The colorimetric assay for the activity of pyruvate dehydrogenase (EC 1.2.4.1) developed by Itokawa is based on a coupled reduction of ferricyanide and the formation of Prussian blue (*Brian Research* 94, 475–484). In this assay system, we found that the coenzyme, thiamine pyrophosphate, itself reduced ferricyanide independent of both the enzyme and the substrate. Similar effect was also observed with thiamine, but not with thiochrome. The reduction of ferricyanide by thiamine or thiamine pyrophosphate was blocked by trichloroacetic acid. Measurement of the activity of purified pyruvate dehydrogenase based on monitoring the reduction of ferricyanide as described by Schwartz et al (*Biochem. Biophys. Res. Commun.* **31,** 495–500) has been widely used. Our findings clearly disprove the assumption that reduction of ferricyanide is dependent on pyruvate dehydrogenase and invalidate assays for thiamine-dependent dehydrogenases based on the reduction of ferricyanide.

The pyruvate dehydrogenase complex (PDC) (EC 1.2.4.1 + EC 2.3.1.12 + EC 1.8.1.4) catalyzes the oxidative decarboxylation of pyruvate, using thiamine pyrophosphate (TPP), CoA and NAD^+ as coenzymes[(1)]. Three products, namely acetyl-CoA, CO_2 and NADH are formed during the decarboxylation of pyruvate, and quantification of these products can be used for the assay of the enzyme activity. Formation of NADH can be measured by monitoring its characteristic absorbance at 340 nm[(2-6)]. Measurement of CO_2 released is facilitated by using [1-^{14}C] pyruvate as substrate[(7)]. Formation of acetyl-CoA is measured by coupling with an arylamine acetyltransferase-catalyzed reaction[(6)] or with a non-enzymatic O-acylation of excess dithioerythritol[(8)]. In place of NAD^+, other electron accepting chemicals can be used for the colorimetric assay of the enzyme activity. For example, Itokawa[(9)] coupled the reduction of ferricyanide with the activity of pyruvate dehydrogenase, and the activity was calculated based on the formation of ferrocyanide as measured by the intensity of Prussian blue[(10)] formed after ferric chloride was added.

We have attempted to measure the activity of pyruvate dehydrogenase complex in chicken brain and liver homogenates following the Itokawa method. In an additional set of control of TPP inclusion without any enzyme source, a step which was not included in the original protocol, Prussian blue formation was observed upon the addition of ferric chloride. Thus TPP reduced ferricyanide independent of enzymatic activity. Further more, reduction of ferricyanide by TPP or thiamine was independent of pyruvate. This independence of both enzyme and substrate questions the validity of the assay methods of TPP-dependent dehydrogenases based on the reduction of ferricyanide.

MATERIALS AND METHODS

Thiamine pyrophosphate (TPP) chloride, thiamine hydrochloride, thiochrome, sodium pyruvate and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co. Potassium ferricyanide and potassium ferrocyanide were

[1] This study was supported in part by a Hatch grant from the College of Agricultural and Life Sciences, University of Wisconsin-Madison.

[2] Corresponding author. Fax: 1-608-262-6005. Email: wdwu@calshp.cals.wisc.edu.

0006-291X/96 $18.00

TABLE I
Absorbance of Prussian Blue Formed in the Experiment with Chicken Brain Homogenate

Tube #	TPP added μmoles	Enzyme extract added (μl) 0	20	40	60	80	100	120	140	160
1	—	0.1061[1]	0.1201	0.1448	0.1996	0.2013	0.2194	0.2527	0.3742	0.3025
2	—	0.1131	0.2500	0.4700	0.6312	0.6840	0.7800	0.9050	0.9992	1.2250
3	0.2	1.1619	1.2051	1.2873	1.4397	1.5072	1.5840	1.7370	2.0330	2.0925
Differences between blanks and samples										
Background		0.0000	0.014	0.0387	0.0935	0.0952	0.1133	0.1466	0.1681	0.1914
Enzymatic without TPP		0.0000	0.1369	0.3569	0.5181	0.5709	0.6669	0.7919	0.8861	1.1119
Enzymatic with TPP		0.0000	0.0432	0.1254	0.2778	0.3453	0.4221	0.5751	0.8712	0.9306

[1] Data are the means of duplicate samples.

purchased from Merck and Co. Inc. Ferric chloride and cuvette were purchased from Fisher Scientific Co. Spectrophotometer (DU Series 600 model) of Beckman Co. was used.

Four broiler birds (average body weight of 300 g) were euthanized by CO_2. The brain and liver were excised, weighed, rapidly transferred to ice-cold 50 mM potassium phosphate buffer (pH 6.5) and homogenized with a Polytron homogenizer of Brinkmann Instruments. The ratio of tissue to buffer was 1:5 (w/v). The homogenates were measured for pyruvate dehydrogenase activity following the protocol of Itokawa, based on the following reaction equation:

$$CH_3COCOOH + 2Fe^{+3}(CN)_6 + H_2O \rightarrow CH_3COOH + CO_2 + 2Fe^{+2}(CN)_6 + 2H^+$$

Each assay was carried out in a set of 3 tubes. The first tube served as zero time control. The second tube served as original activity of pyruvate dehydrogenase and the third tube for TPP-stimulated activity. The basic reaction mixture contained 150 μmoles of potassium phosphate, 1.0 μmole of calcium chloride, enzyme source (0, 20, 40, 60, 80, 100, 120, 140 and 160 μl of tissue homogenate), and 0.2 μmoles of TPP for the TPP series in a final volume of 2.8 ml. The mixtures were preincubated at 37°C for 15 min. Then 10 μmoles of potassium ferricyanide (100 μl) and 50 μmoles of sodium pyruvate (100 μl) were added. To tube 1, 1 ml of 10% was added to stop any enzymatic reaction, and TCA was added to tube 2 and tube 3 after a second incubation at 37°C for 15 min. All the tubes were centrifuged at 500 *g* for 10 min to remove the precipitated protein. Two milliliters of supernatant and 3.3 μmoles of

TABLE II
Absorbance of Prussian Blue Formed in the Experiment with Chicken Liver Homogenate

Tube #	TPP added μmoles	Enzyme extract added (μl) 0	20	40	60	80	100	120	140	160
1	—	0.1019[1]	0.1308	0.1675	0.2001	0.2274	0.2466	0.2806	0.3121	0.3287
2	—	0.1124	0.3504	0.5197	0.7891	1.0259	1.1853	1.2570	1.6580	1.7443
3	0.2	1.2847	1.3730	1.5197	1.5641	1.6367	1.7145	1.9274	2.1245	2.3526
Differences between blanks and samples										
Background		0.0000	0.0309	0.0656	0.0982	0.1255	0.1447	0.1787	0.2102	0.2268
Enzymatic without TPP		0.0000	0.238	0.4073	0.6767	0.9135	1.0729	1.1446	1.5456	1.6319
Enzymatic with TPP		0.0000	0.0883	0.235	0.2794	0.352	0.4298	0.6427	0.8398	1.0679

[1] Data are the means of duplicate samples.

TABLE III
Effects of TPP, Thiamine, Pyruvate and TCA on the Formation of Prussian Blue

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8
	μmoles added							
Buffer	150	150	150	150	150	150	150	150
TPP	—	—	0.2	0.2	0.2	—	—	—
Thiamine	—	—	—	—	—	0.2	0.2	0.2
Pyruvate	50	50	50	50	—	50	50	—
Ferricyanide	10	10	10	10	10	10	10	10
TCA[1]	*before*	*after*	*before*	*after*	*after*	*before*	*after*	*after*
$FeCl_3$	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3
	OD/660nm							
Mean (n = 3)								
± SD	0.0351	0.0732	0.1237	1.0676	1.0921	0.1459	0.6910	0.7120
	0.0107	0.0102	0.0207	0.0067	0.0234	0.0080	0.0090	0.008

[1] TCA (10%, 1 ml) was added *before* or *after* the second incubation.

$FeCl_3$ (10 μl) were mixed directly in a cuvette, optical density at 660 nm was measured after 10 min but before 15 min at room temperature.

In the following non-enzymatic investigation, reaction mixtures were constituted basically following the enzymatic study, but the enzyme source was excluded. The chemical mixtures in the 8 series of tubes are indicated in Table III. The order of addition of each chemical was followed by the sequence in column 1 of the table.

To test the theory that the dissociable proton of the reactive carbon of the thiazole ring of thiamine is required for the reduction of ferricyanide, thiochrome (oxidized thiamine) was also tested for the ability to reduce ferricynide in an experiment set up as indicated in Table IV.

TABLE IV
Effect of Thiochrome on the Formation of Prussian Blue

	Tube 1	Tube 2	Tube 3
	μmoles added		
Buffer	150	150	150
TPP	—	0.2	—
Thiochrome[1]	—	—	0.2
Methanol	500	500	500
Ferricyanide	10	10	10
TCA[2]	+	+	+
$FeCl_3$	3.3	3.3	3.3
	OD/660nm		
Mean (n = 3)			
± SD	0.0196	1.7353	0.3650
	0.0102	0.0104	0.0205

[1] Thiochrome was initially dissolved in 0.1 ml methanol, diluted to 0.5 ml with H_2O, and 100 μl was added per tube.

[2] TCA (10%, 1 ml) was added 15 minutes after other chemicals have been mixed and incubated.

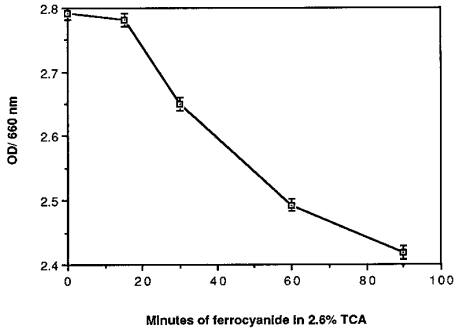

FIG. 1. Stability of ferrocyanide in 2.6% TCA. Fifty μmoles of potassium ferrocyanide was dissolved in 14 ml of phosphate buffer and mixed with 5 ml of 10% TCA, left at 22°C from 0 to 90 min, then measured for Prussian blue formation immediately after mixing 2 ml of potassium ferrocyanide-TCA solution with 10 μl (3.3 μmoles) of ferric chloride. n=3, vertical bar=SD.

To test the stability of ferrocyanide in TCA, 50 μmoles of ferrocyanide was dissolved in 14 ml of phosphate buffer, mixed with 5 ml of 10% TCA, and left at room temperature for 0 to 90 min. At a given time, ferrocyanide in solution was monitored by reading the optical density due to Prussian blue at 660 nm immediately following the mixing of two ml of mixture with 3.3 μmoles of $FeCl_3$.

RESULTS

Results of enzymatic assays using brain or liver tissue homogenates are presented in Tables I and II respectively. For both tissue types, gradually increased amount of enzyme (volume of tissue homogenate) caused corresponding increases in absorbance due to the background, and due to the apparent enzymatic activity in the presence or absence of exogenous TPP. Whether this apparent enzymatic activity could be ascribed to the action of pyruvate dehydrogenase becomes questionable when 10 time increase of OD due to the addition of TPP alone (no amount of enzyme added) was observed. When the enzymatic differences of OD were calculated between addition and non-addition of TPP, TPP appeared to inhibit the activity of pyruvate dehydrogenase.

The effects of TPP, thiamine, pyruvate, and TCA on the reduction of ferricyanide and thereby Prussian blue formation, are presented in Table III. Both TPP and thiamine reduced ferricyanide (tube 4 and tube 7), which was inhibited by the prior addition of TCA. On an equal mole basis, TPP reduced more ferricyanide than thiamine (by 55%). Reduction of ferricyanide by either TPP or thiamine was independent of pyruvate (tube 5 and tube 8). No Prussian blue was formed when TPP was substituted with thiochrome (Table IV). The amount of ferrocyanide in 2.6% TCA decreased drastically 15 min after it was initially dissolved in the acid (Figure 1).

DISCUSSION

Thiamine pyrophosphate is a required coenzyme of pyruvate dehydrogenase and forms an intermediate with pyruvate through the reactive carbon atom between the nitrogen and sulfur atoms in the thiazole ring[1]. This carbon is much more acidic than most =CH- groups (Fig. 2), which can deprotonate, form a carbanion, and then donate an electron to ferricyanide, with a concomitant reduction of ferricyanide. As indicated in Figure 2, electron transfer from this carbon to ferricyanide would prohibit the formation of TPP-pyruvate intermediate, resulting in the observed inhibition of apparent enzymatic activity. TCA is a strong organic acid, and in its presence, the dissociable proton of the reactive carbon atom is stable and the carbon

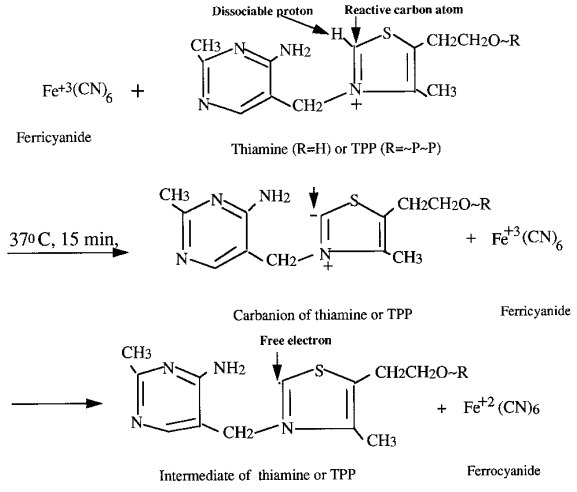

FIG. 2. Schematic diagram of oxidation of thiamine or TPP.

atom can not ionize to form a carbanion, thus blocks the transfer of an electron from the reactive carbon of thiazole ring to ferricyanide (Fig. 3). This reactive carbon atom can also catalyze the non-enzymatic decarboxylation of pyruvate at slightly alkaline condition (pH 8.9)[11], and is the site being oxidized by $HgCl_2$ or ferricyanide under strong alkaline conditions, with the formation of thiochrome[12,13]. The requirement for the presence of this reactive carbon for the reduction of ferricyanide by thiamine and TPP is substantiated by using thiochrome in which that particular carbon can not form carbanion (Fig. 4). Using an established method[13], thiochrome was not found in our systems before the TCA addition. However, we have made no effort to examine the possible structure change of thiamine or TPP in our systems. The observed unstable nature of ferrocyanide in TCA agreed with the previous observation[14]. Therefore, the determination of ferrocyanide as Prussian blue formation has to be carried out as soon as possible after the protein was precipitated.

Prior to Itokawa's description of his colorimetric method, Schwartz et al. developed a routine assay for purified pyruvate dehydrogenase by monitoring the coupled reduction of fericyanide at 420 nm[15]. Schwartz method has been widely used in many laboratories to measure the activity of TPP-dependent pyruvate dehydrogenase[16-22]. Both Itokawa and Schwartz methods assumed the dependence of ferricyanide reduction on the activity of the TPP-dependent enzyme.

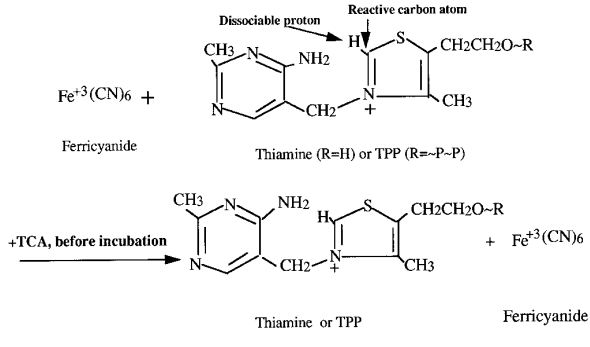

FIG. 3. Schematic diagram of the prohibition of oxidation of thiamine or TPP by TCA.

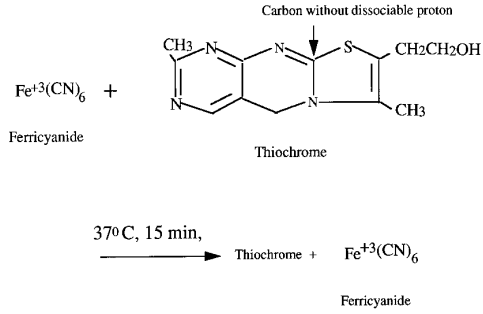

FIG. 4. Schematic diagram of oxidation of thiochrome.

Our findings of the non-dependence of both enzyme and substrate of reduction of ferricyanide by TPP invalidate any assays using ferricyanide as a coupling reagent when TPP has to be included.

REFERENCES

1. Stryer, L. (1988) Biochemistry: Citric Acid Cycle, 3rd ed., pp. 374–378, W. H. Freeman and Company, New York.
2. Linn, T. C., Pelley, J. W., Pettit, F. H., Hucho, F., Randall, D. D., and Reed, L. J. (1972) *Arch. Biochem. Biophys.* **148,** 327–342.
3. Visser, J., Kester, H., Jeyaseelam, K., and Topp, R. (1982) *in* Methods in Enzymology (Wood, W. A., Ed.), Vol. 89, pp. 399–407, Academic Press, New York/London.
4. Randall, D. D. (1982) *in* Methods in Enzymology (Wood, W. A., Ed.), Vol. 89, pp. 408–414, Academic Press, New York/London.
5. Brown, J. P., and Perham, R. N. (1976) *Biochem. J.* **155,** 419–427.
6. Furuta, S., and Hashimoto, T. (1982) *in* Methods in Enzymology (Wood, W. A., Ed.), Vol. 89, pp. 414–416, Academic Press, New York/London.
7. Paxton, R., and Sievert, L. M. (1991) *Biochem. J.* **277,** 547–551.
8. Liedvogel, B. (1985) *Anal. Biochem.* **148,** 182–189.
9. Itokawa, Y. (1975) *Brain Research.* **94,** 475–484.
10. Greenwood, N. N., and Gibb, T. C. (1971) Mossbauer Spectroscopy, pp. 169–178, Chapman and Hall Ltd., London.
11. Yatco-Manzo, E., Frances, R., Yount, R. R., and Metzler, D. (1958) *J. Biol. Chem.* **234,** 733–737.
12. Edwin, E. E. (1979) *in* Methods in Enzymology (McCormick, D. B., and Wright, L. D., Eds.), Vol. 62, pp. 51–54, Academic Press, New York/London.
13. Penttinen, H. K. (1979) *in* Methods in Enzymology (McCormick, D. B., and Wright, L. D., Eds.), Vol. 62, pp. 58–59, Academic Press, New York/London.
14. American Cyanamid Co., (1953) The Chemistry of the Ferrocyanides, p. 32, Beacon Press, New York.
15. Schwartz, E. R., Old, L. O., and Reed, L. J. (1968) *Biochem. Biophys. Res. Commun.* **31,** 495–500.
16. Snoep, J. L., Demattos, M. J. T., Postma, P. W., and Neijssel, O. M. (1990) *Arch. Microbio.* **154,** 50–55.
17. Graupe, K., Trommer, W. E., and Bisswanger, H. (1989) *Biochim. Biophys. Acta* **999,** 176–182.
18. Madhosingh, C., and Orr, W. (1985) *J. Environ. Sci. Health Part B.* **20,** 201–214.
19. Sumegi, B., and Alkonyi, I. (1983) *Arch. Biochem. Biophys.* **223,** 417–424.
20. Visser, J., and Strating, M. (1982) *in* Methods in Enzymology (Wood, W. A., Ed.), Vol. 89, pp. 391–399, Academic Press, New York/London.
21. Adamson, S. R., and Stevenson, K. J. (1981) *Biochem.* **20,** 3418–3424.
22. O'Brien, T. A., and Gennis, R. B. (1980) *J. Biol. Chem.* **255,** 3302–3307.