REDUCTION OF DEHYDROASCORBIC ACID WITH PYRUVATE OXIDASE SYSTEM

II. REDUCTION OF DEHYDROASCORBIC ACID WITH PYRUVATE OXIDASE ISOLATED FROM PIG HEART MUSCLE¹

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It was previously reported by the authors (1) that, as long as pyruvate exists in the medium containing *E. coli* K₁₂, dehydroascorbic acid is reduced, and this reducing activity is dependent upon a pyruvate oxidase system. The presence of the mechanism of reducing dehydroascorbic acid into ascorbic acid in the bacterial cells would serve the regulation of ascorbic acid, protecting its decomposition, because, once dehydroascorbic acid is delactonized, it is easily decomposed under oxidation. As dehydroascorbic acid is delactonized by the enzymatic reaction in animal tissues (2-3), the presence of the mechanism of reducing dehydroascorbic acid into ascorbic acid in animal tissues might be desirable.

The present report describes the mechanism of the reduction of dehydroascorbic acid with the pyruvate oxidase isolated from pig heart muscle.

EXPERIMENTAL

Materials and Methods

1. Chemicals

Reduced NAD and CoA were obtained from C. F. Boehringer and Sohne Company, Ltd., and lipoamide from Fujisawa Pharmaceutical Co. Other materials were also obtained commercially. L-Ascorbic acid used was recrystallized from methanol. Dehydroascorbic acid solution was obtained by the oxidation of ascorbic acid solution with bromine, the small excess of which was removed by vigorous aeration, and used within 5 minutes.

2. Preparation of Pyruvate Oxidase

The partial purification of pyruvate oxidase from the pig heart muscle was carried out by the method of Korkes (4).

¹ Following abbreviations are used: AsA, L-ascorbic acid; DAsA, dehydro-L-ascorbic acid; TDP, thiamine diphosphate; LiA, lipoic acid; LiAH₂, dihydrolipoic acid; LiANH₂, lipoamide.

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3. Assay Method of the Activity of Reducing Dehydroascorbic Acid Using Pyruvate as a Hydrogen Donor

Dehydroascorbic acid (2.0 μ moles) was incubated in a Thunberg tube with the pyruvate oxidase, sodium pyruvate (4.0 μ moles), TDP (1.0 μ moles), CoA (0.1 μ mole), GSH (0.5 μ mole), MgSO_4 (10.0 μ moles), LiANH_2 (1.0 μ moles) and phosphate buffer, pH 6.8, (100.0 μ moles) in the final volume of 4.0 ml under anaerobic condition at 37° for 10 minutes. Dehydroascorbic acid should be added last of all. The reaction was stopped by the addition of 1.0 ml of 20 % metaphosphoric acid at 0°, and the mixture was filtered. The remaining dehydroascorbic acid and the total ascorbic acid in the filtrate were determined as presented in the previous paper (1). The difference between total ascorbic acid and dehydroascorbic acid was assumed to be the amount of ascorbic acid formed.

4. Assay Method of the Activity of Reducing Dehydroascorbic Acid Using Reduced NAD as a Hydrogen Donor

Dehydroascorbic acid (2.0 μ moles) was incubated in a Thunberg tube with pyruvate oxidase, reduced NAD (6.0 μ moles), LiANH_2 (1.0 μ moles) and phosphate buffer, pH 7.0, (100.0 μ moles) in the final volume of 2.0 ml under anaerobic condition at 37° for 10 minutes. The reaction was stopped by the addition of metaphosphoric acid at 0°. The amount of ascorbic acid formed was estimated as described above.

RESULTS

1. Reduction of Dehydroascorbic Acid with Pyruvate Oxidase

The activity of reducing dehydroascorbic acid with the pyruvate oxidase isolated from pig heart muscle was determined (Table 1).

TABLE 1
Reduction of Dehydroascorbic Acid with Pyruvate Oxidase

	AsA formed
	μ moles
Complete system	1.40
— TDP	0.31
— LiANH_2	0
— CoA	0
— Pyruvate	0
— Pyruvate oxidase	0

Experimental conditions: DAsA (2.0 μ moles) was incubated with pyruvate oxidase (1.5 ml), sodium pyruvate (4.0 μ moles), TDP (1.0 μ mole), CoA (0.1 μ mole), GSH (0.5 μ mole), MgSO_4 (10.0 μ moles), LiANH_2 (1.0 μ mole) and phosphate buffer, pH 6.8, (100.0 μ moles) in the final volume of 4.0 ml under anaerobic condition at 37° for 10 min.

In this system, TDP, LiANH_2 , CoA and pyruvate were essential for the reduction of dehydroascorbic acid.

2. Effect of Reduced NAD on the Activity of Reducing Dehydroascorbic Acid with Pyruvate Oxidase

Dehydroascorbic acid was reduced in the presence of pyruvate oxidase using reduced NAD as a hydrogen donor (Table 2).

TABLE 2
Effect of Reduced NAD on the Activity of Reducing Dehydroascorbic Acid with Pyruvate Oxidase

	AsA formed
	μmoles
Complete system	1.20
— LiANH ₂	0.11
— Reduced NAD	0
— Pyruvate oxidase	0

Experimental conditions: DAsA (2.0 μmoles) was incubated with pyruvate oxidase (1.5 ml), reduced NAD (6.0 μmoles), LiANH₂ (1.0 μmole) and phosphate buffer, pH 7.0, (100.0 μmoles), in the final volume of 2.0 ml under anaerobic condition at 37° for 10 min.

In this system, a catalytic amount of LiANH₂ was essential for the reduction of dehydroascorbic acid as hydrogen acceptor.

3. Stoichiometry

The reaction assayed under the standard condition proceeded almost stoichiometrically (Table 3).

TABLE 3
Stoichiometry

Exp. No.	Hydrogen donor	DAsA added	AsA formed	DAsA remained
		μmoles	μmoles	μmoles
1 ^a	Pyruvate	2.0	1.40	0.58
2 ^a	Pyruvate	2.0	1.35	0.63
3 ^b	Reduced NAD	2.0	1.09	0.89
4 ^b	Reduced NAD	2.0	1.21	0.79

^a The experimental conditions were the same as those presented in Table 1.

^b The experimental conditions were the same as those presented in Table 2.

4. Reaction Product

Dehydroascorbic acid, reduced NAD and LiANH₂ (10 times the standard condition) were incubated with excess pyruvate oxidase until the remaining dehydroascorbic acid was not detectable at all with 2,4-dinitrophenylhydrazine, and then

TABLE 4
Thin Layer Chromatography of Reaction Product

	R _f value using the developing solvent system		
	A	B	C
Dinitrophenylhydrazine-treated oxidation product	0.15	0.28	0.48
Dinitrophenylhydrazine derivative of DAsA	0.14	0.28	0.47

A, cyclohexane-acetone (7:3); B, chloroform-acetone (9:1); C, toluene-acetone-5% acetic acid (2:1:1).

the reaction mixture was deproteinized by metaphosphoric acid. The product was oxidized to dehydroascorbic acid with bromine, a small excess of which was removed by the addition of thiourea. The oxidation product was treated with 0.4 % 2,4-dinitrophenylhydrazine in 1 *N* H₂SO₄ at 50° for 1 hour. The precipitate obtained was washed with distilled water, and recrystallized from hot methanol. The *R_f* values of bis-hydrazone in thin layer chromatography are shown in Table 4. There was no difference in the *R_f* values of bis-hydrazone between the oxidation product and dehydroascorbic acid.

5. Reduction of Dehydroascorbic Acid with Dihydrolipoic Acid

In an effort to determine the mechanism of reduction of dehydroascorbic acid using pyruvate oxidase, the activity of the enzyme which catalyzes the reaction of the hydrogen transfer from LiAH₂ to dehydroascorbic acid was studied. LiAH₂ was added to the medium containing pyruvate oxidase, and the whole was incubated at 37°. As the non-enzymatic reduction of dehydroascorbic acid by LiAH₂ was extremely rapid, a participation of an enzyme was not determined.

6. Stoichiometry of the Reaction of the Non-enzymatic Reduction of Dehydroascorbic acid with Dihydrolipoic Acid

The non-enzymatic reduction of dehydroascorbic acid with LiAH₂ proceeded almost stoichiometrically (Table 5).

TABLE 5
Stoichiometry of the Non-Enzymatic Reduction of Dehydroascorbic Acid with Dihydrolipoic Acid

Exp. No.	DAsA added	AsA formed	DAsA remained	LiAH ₂ oxidized
	<i>μmoles</i>	<i>μmoles</i>	<i>μmoles</i>	<i>μmoles</i>
1	2.0	1.1	0.9	1.0
2	2.0	1.0	1.0	1.0

Experimental conditions: DAsA (2.0 *μmoles*) was incubated with LiAH₂ and phosphate buffer, pH 6.8, (100.0 *μmoles*) in the final volume of 4.0 ml under anaerobic condition at 37° for 3 min.

LiAH₂ was determined by the method of Nishida (9).

DISCUSSION

It has been known that an enzyme catalyzing the reduction of dehydroascorbic acid by glutathione to ascorbic acid occurs in plant tissues. Recently, it was reported by Hughes that the reduction of dehydroascorbic acid by the enzyme isolated from guinea pig liver occurs at a rate greater than can be accounted for in term of a purely chemical reduction by glutathione (5).

The authors (6) first observed dehydroascorbic acid reductase in *E. coli* K₁₂. The enzyme was effective on the hydrogen donors such as glutathione, homocysteine, thioglycolate and cysteine, but was not on reduced NAD, CoA and LiAH₂. The authors observed that, as long as pyruvate exists in the medium containing *E. coli* K₁₂, dehydroascorbic acid is reduced, and this reducing activity was independent of dehydroascorbic acid reductase. In this system, LiAH₂ was the hydrogen donor, and dehydroascorbic acid is assumed to be reduced into ascorbic acid by the

reaction sequences given in Fig. 4 of the foregoing paper (1).

Rosenberg (7) reported that scurvy in the guinea pig was slightly prevented by the administration of LiA. The present paper clarified the presence of a reducing mechanism of dehydroascorbic acid into ascorbic acid with the pyruvate oxidase isolated from pig heart muscle. The mechanism was concluded to be similar to that obtained from *E. coli* K_{12} as described above.

In animals, dehydroascorbic acid is enzymatically delactonized to 2,3-diketogulonic acid (2-3), which cannot become dehydroascorbic acid by the reverse reaction. Therefore, 2,3-diketogulonic acid lacks the antiscorbutic activity, whereas dehydroascorbic acid possesses the activity, and what is eliminated in the urine following excess ascorbic acid administration is not dehydroascorbic acid, but ascorbic acid (8). Therefore, the presence of the mechanism of reducing dehydroascorbic acid into ascorbic acid might be desirable for the animals.

SUMMARY

1. As long as pyruvate existed in the medium containing the pyruvate oxidase isolated from pig heart muscle, dehydroascorbic acid was reduced, and the reducing activity was independent of dehydroascorbic acid reductase.

2. The reducing activity was dependent upon a pyruvate oxidase system, and the hydrogen donor was found to be dihydrolipoic acid.

3. As the non-enzymatic reduction of dehydroascorbic acid with dihydrolipoic acid was extremely rapid, participation of an enzyme catalyzing the hydrogen transfer from dihydrolipoic acid to dehydroascorbic acid was not determined.

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