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RATE ENHANCEMENT OF THE ELECTRON TRANSFER OF THE ADRENODOXIN-ADRENODOXIN REDUCTASE SYSTEM BY DICARBOXYLIC ACIDS

Toshio Yamano[1], Yasuki Nonaka, Mitsuhiro Okamoto, Takashi Matsubara*, and Retsu Miura**

Departments of Biochemistry and Molecular and Physiological Chemistry, Osaka University Medical School, Kita-ku, Osaka 530, Japan

*Shionogi Research Laboratories, Shionogi and Co., Ltd., Fukushima, Osaka 553, Japan

**Laboratory of Chemistry, Kansai Medical University, Hirakata 573, Japan

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Summary: The rate of electron transport in the cytochrome P-450 system in adrenocortical mitochondria was studied with purified adrenodoxin reductase, adrenodoxin and cytochrome c. Oxaloacetate enhanced the rate at concentrations of less than 1 mM; malate, succinate and fumarate enhanced the rate to a lesser extent; and pyruvate and α-ketoglutarate had no appreciable effect. The rate enhancement was observed when the reagents were preincubated with adrenodoxin, but not with adrenodoxin reductase. Rate enhancement was also evident when the rate limiting step was at adrenodoxin in the electron transport system.

Both adrenodoxin(1) and NADPH:adrenodoxin oxidoreductase (EC.1.18.1.2, adrenodoxin reductase)(2,3) from bovine adrenocortical mitochondria have been purified and crystallized. Their primary structures have been determined by chemical analysis(4) or molecular cloning(5) for the former and by cloning of the latter(6,7). By the use of these pure samples, the kinetics of the electron transport from the reductase to adrenodoxin and from adrenodoxin to the cytochrome P-450's or to cytochrome c instead of the P-450's have been extensively studied.

Although cytochrome c is not a physiological electron acceptor, the high rate of the reduction of cytochrome c by reduced adrenodoxin makes it feasible to analyze the factors that may affect the rate of electron transport in the reduction or reoxidation of

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adrenodoxin, because experiments can be so designed as to shift the rate limiting step from the step between adrenodoxin and cytochrome c to other steps.

As it is well-known that complex formation occurs between adrenodoxin and the reductase(8,9), there may not be any factor that changes the intrinsic electron transport per se between these two proteins. Though a high ionic strength reduces the rate of electron transport, it is regarded to do so by making the complex dissociable and not by affecting the intrinsic rate constant of the electron transport.

In this communicate, we report that dicarboxylic acids such as oxaloacetate affect the electron transfer rate of the system at concentrations below 1mM.

MATERIALS and METHODS

Adrenodoxin reductase was isolated from bovine adrenocortical mitochondria and purified to the crystalline state according to the procedure of Nonaka et al.(10). It was kept frozen at -80℃ in the form of a crystalline suspension at a concentration of about 0.6 mM. A small amount of the preparation was taken from the stock, and it was then diluted 10 times with Tris-HCl buffer and used for the experiments after further dilution to the appropriate concentrations. The ten-fold diluted solution was stored at -20℃ until use. The activity was measured optically by means of the reduction rate of 0.9 mM ferricyanide at 25 ℃. The concentration of the reductase was estimated from the molecular activity of the reductase of 10.96/sec/FAD of the reductase (11), taking the molar extinction coefficient of ferricyanide as 1020 at 420 nm (12). The concentration of the reductase was also confirmed by using its molecular absorbance at 450 nm: 10,900 M^{-1} cm^{-1}(13). Adrenodoxin was crystallized according to the procedure of Ohnishi et al. (14). The ratio of A_{414} / A_{276} was 0.90. The crystalline suspension (about 0.9 mM) was also kept frozen at -80℃. It was diluted 10 times as a transient stock solution, kept at -20 ℃ and used for the experiments after further dilution. The concentration of adrenodoxin was determined from the absorbance at 414 nm, using the molecular absorbance value of 11 mM^{-1} cm^{-1} (15). Type V cytochrome c from bovine heart was purchased from Sigma Co.; and its concentration was determined by the difference molar extinction coefficient, 19 mM^{-1} cm^{-1}, between the enzymatically reduced and the oxidized cytochrome c (16). NADPH was obtained from the Oriental Yeast Co.,Ltd., Japan. Other reagents were of analytical grade. Spectrophotometric measurements were determined with a Hitachi U 3200 spectrophotometer with digital output and graphic print-out. A time scan of the electron transport reaction from NADPH to cytochrome c via adrenodoxin and the reductase was recorded at 550 nm. The reaction was carried out at 37℃ at pH 7.4, unless otherwise specified. A cuvette of 1 cm light-path was used in a thermostated cell holder. The reaction was started by adding the last reagent, NADPH, from a microsyringe and stirring with a micromotor-drived mixer.

RESULTS and DISCUSSION

Enhancement of the electron transfer rate by oxaloacetate. Incubation of adrenodoxin with oxaloacetate for a few minutes enhanced the rate of electron transfer from NADPH to cytochrome c via adrenodoxin and the reductase. From the time course of the increase in the absorbance of reduced cytochrome c at 550 nm, the rates of electron transfer were obtained. The activity was defined as the increase in absorbance in the first minute after initiation of the reaction, a period in which the increase was nearly linear with time. The percent activity was expressed as the ratio of the activity with adrenodoxin pre-incubated with oxaloacetate to that with adrenodoxin without oxaloacetate. The dependence of the activity on the concentration of oxaloacetate leveled-off at about 0.5 mM. Oxaloacetate was neutralized in the aquaeous solution by NaOH or in the Tris HCl buffer. Care was taken to prevent the degradation of oxaloacetate, which is quite unstable. Storage of oxaloacetate dissolved in Tris buffer produced a species that showed decreased absorption at 230 nm

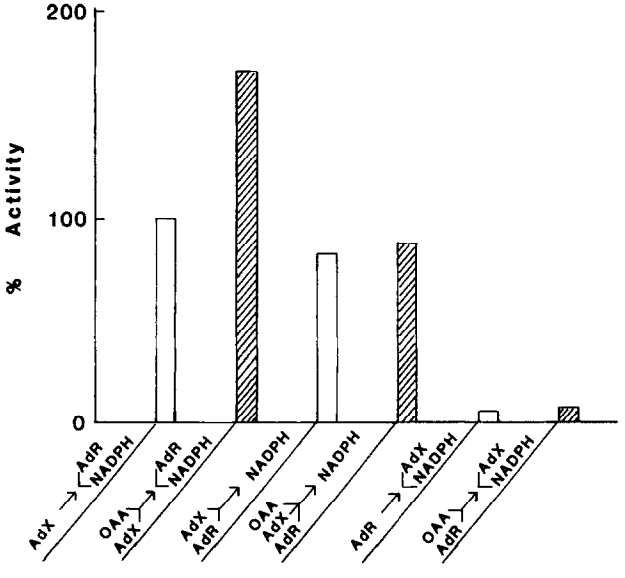

Fig. 1. Effect of oxaloacetate(OAA) on the cytochrome c reduction by the adrenodoxin(AdX)-adrenodoxin reductase(AdR) system. The arrows denote the two-minute incubation prior to the initiation of the reaction by addition of NADPH. Superposition of reagent letters denote the immediate mixing of two or three reagents. Hatched boxes indicate the addition of oxaloacetate in comparison with the left open boxes, in which oxaloacetate was not added. Concentrations in a total volume of 2.3 ml were: cytochrome c, 15 uM; adrenodoxin, 4.5 nM; adrenodoxin reductase, 40 nM; NADPH, 50 uM; oxaloacetate, 1 mM when added; Tris-HCl, pH 7.4, 25 mM. The reaction temperature was 37 °C.

and different absorption spectrum from either oxaloacetate or pyruvate; such a solution showed markedly reduced ability to enhance the rate of electron transport. The incubation time was changed at a definite concentration of oxaloacetate. It turned out that the enhancing effect of oxaloacetate was achieved within half a minute of incubation. While the rate enhancement was observed with pre-incubation of oxaloacetate with adrenodoxin, the complex formation of adrenodoxin with the reductase achieved by co-incubation for two minutes canceled the effect of oxaloacetate, as seen in Fig.1. In contrast to the pre-incubation of oxaloacetate with adrenodoxin, pre-incubation of oxaloacetate with adrenodoxin reductase failed to enhance the rate, while the activity of the reductase itself decreased due to the dissocition of FAD from the reductase during the pre-incubation with or without oxaloacetate (manuscript in preparation). Moreover, it was also demonstrated that the reductase activity of ferricyanide reduction was not affected by oxaloacetate (data not shown).

Enhancement of the electron transfer rate by the various dicarboxylic acids. As shown in Fig. 2, a series of dicarboxylic acids were found to be effective for enhancing the rate to different extents, oxaloacetate being the most effective among them at the same concentration. To analyze the active residues of oxaloacetate, α-ketoacid, pyruvate or α-ketoglutarate was used

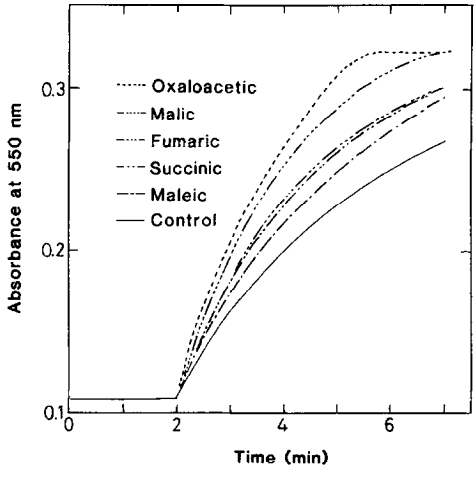

Fig. 2. Time course and rate enhancement by dicarboxylic acids of the cytochrome c reduction via the adrenodoxin-adrenodoxin reductase system. The concentrations of dicarboxylic acids used were 1 mM. The reaction conditions were the same as those in Fig. 1. The reactions were started by the simultaneous addition of adrenodoxin reductase and NADPH.

to determine whether the carbonyl residue or dicarboxylic residues in the rate enhancement. Pyruvate or α-ketoglutarate showed little ability to enhance the rate (data not shown). According to these results, it is unlikely that rate enhancement occurs through a mechanism involving Schiff base formation between the amino group of adrenodoxin and oxaloacetate. Instead, properly oriented dicarboxylic residues may be involved through an interaction with adrenodoxin.

When adrenodoxin was incubated with oxaloacetate or malate prior to the reaction and an appropriate-sized aliquot of the mixture was put in the optical cell to give the same final concentration of adrenodoxin as in Fig. 2 but a diluted concentration of enhancer, there was no enhancement of the rate of electron transfer. This result also favors the mechanism of non-covalent interaction between adrenodoxin and oxaloacetate.

The rate enhancement of oxaloacetate is only observable when the rate limiting step is located between adrenodoxin and the reductase. As shown in Fig. 3, rate enhancement in the presence of oxaloacetate was seen when the concentration of the reductase was in excess and the rate of reduction of cytochrome c was dependent on the concentration of adrenodoxin. On the contrary, when the concentration of the reductase was low and the rate of cytochrome c reduction was dependent on the reductase

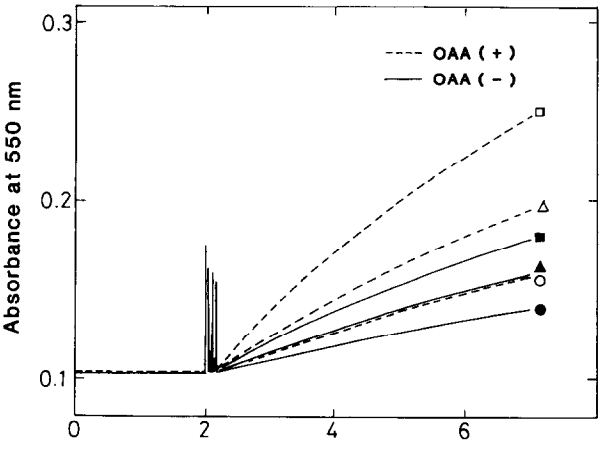

Fig. 3. Time courses of the cytochrome c reduction via the adrenodoxin-adrenodoxin reductase system with or without oxaloacetate at different concentrations of adrenodoxin at a saturating level of adrenodoxin reductase. Concentrations were: adrenodoxin reductase, 40 nM; adrenodoxin, 4.5 nM (● and ○), 9 nM(▲ and △), 12 nM (■ and □). Open and closed symbols denote the reactions with and without oxaloacetate, respectively. The other conditions are the same as those in Figs. 1 and 2.

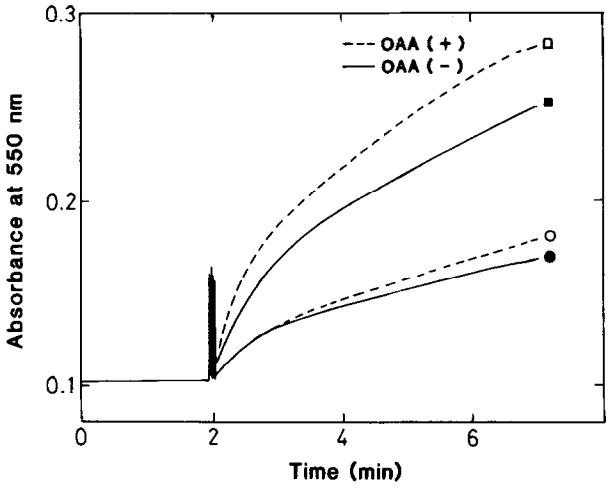

Fig. 4. Time courses of the cytochrome c reduction via the adrenodoxin-adrenodoxin reductase system with or without oxaloacetate at different concentrations at a non-saturating level of adrenodoxin reductase. Concentrations were: adrenodoxin, 40 nM; adrenodoxin reductase, 3.6 nM (● and ○), 7.2 nM (■ and □). Other conditions are as in Figs. 1-3.

concentration, the effect of oxaloacetate on the enhancement remained low, as seen in the lower two curves, with and without oxaloacetate, in Fig. 4, where the concentration of the reductase was less than the saturating level. The enhancement appeared when the concentration of the reductase was increased, as shown in the upper two curves in Fig. 4.

The content of adrenodoxin has been reported to be several times higher than adrenodoxin reductase in adrenocortical mitochondria (17,18). The ratio of adrenodoxin to the reductase used in our experiments was kept low so that the enhancing effect of dicarboxylic acids was observable. Under physiological conditions, however, the status of the occurrence of the two components is more complicated because there are differences in their properties of membrane association. It also remains to be determined whether those dicarboxylic acids have a physiological function, and the molecular mechanism of the enhancement of electron transfer by these agents requires further investigation. In this context, it should be noticed that there was no distinct difference spectrum between adrenodoxin with and without malate.

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