

Rate enhancement of the electron transfer of the adrenodoxin-adrenodoxin reductase system by inorganic and nucleotide phosphates

Toshio Yamano¹, Yasuki Nonaka² and Retsu Miura¹

¹Laboratory of Chemistry, Kansai Medical University, 18-19 Uyahigashi, Hirakata 573 and ²Departments of Biochemistry, and Molecular and Physiological Chemistry, Osaka University Medical School, 4-3-57 Nakanoshima, Kita-ku, Osaka 530, Japan

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Phosphate and pyrophosphate increased the rate of reduction of adrenodoxin by NADPH-adrenodoxin reductase and NADPH, pyrophosphate being one order more effective than the former. However, the cytochrome *c* reduction by the electron transport system was inhibited in the presence of inorganic (pyro)phosphate. On the other hand, ADP and ATP enhanced the rates of reduction of both adrenodoxin and cytochrome *c* through adrenodoxin by the electron transport system. GTP also enhanced the rate of reduction of cytochrome *c* by this system, whereas AMP showed no appreciable enhancement. These inorganic and nucleotide phosphates did not affect the rate of ferricyanide reduction by the reductase.

Adrenodoxin; Adrenodoxin reductase; Adrenocortical mitochondria; Electron transfer; Pyrophosphate; ATP

1. INTRODUCTION

The primary structures of adrenodoxin and adrenodoxin reductase (NADPH:adrenodoxin oxidoreductase, EC 1.18.1.2) have been well established, through chemical analysis of adrenodoxin [1] as well as molecular cloning of adrenodoxin [2] and the reductase [3,4]. Their three dimensional structures have not yet been determined, although a crystallographic study on the space group and unit cell dimensions has been carried out [5]. Through kinetic studies, the firm complex formation between adrenodoxin and the reductase has become well known [6,7]. However, the mechanism underlying the electron transport between adrenodoxin and P-450's remains to be elucidated. We have reported that dicarboxylic acids enhance the rate of electron transfer from NADPH to cytochrome *c* via the reductase and adrenodoxin [8], noting that the negatively charged ligands may affect the capacity of adrenodoxin for electron transfer. In this paper, we report that (pyro)phosphate ions and nucleotide phosphates enhance the rate of reduction of adrenodoxin via the reductase.

2. 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. [5]. It was kept frozen at -80°C in the form of a crystalline suspension, at a concentration of about

0.3 mM. A small amount of the preparation was taken from the stock, diluted 50 times with Tris-HCl buffer and then used for experiments. The enzyme solution was stable for several days, if stored at -20°C . The activity was measured optically as the rate of reduction of 0.9 mM ferricyanide at 25°C . The concentration of the reductase was estimated from the molecular activity of the reductase, 10.96/s/FAD of the reductase [9], based on a molar extinction coefficient of ferricyanide of $1020\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 420 nm [10]. The concentration of the reductase was confirmed from its molecular absorbance at 450 nm, $10900\text{ M}^{-1}\cdot\text{cm}^{-1}$ [11]. Adrenodoxin was crystallized according to the procedure of Ohnishi et al. [12]. The A_{414}/A_{276} ratio was 0.90. The crystalline suspension (about 1.4 mM) was also kept frozen at -80°C . It was diluted 25 times as a transient stock solution, kept at -20°C and used for experiments after further dilution. The concentration of adrenodoxin was determined from the absorbance at 414 nm, using the molecular absorbance value of $11000\text{ M}^{-1}\cdot\text{cm}^{-1}$ [13]. Adrenodoxin exhibits heterogeneity, which has been reported to be due to the proteinase attack during preparation or storage [14]. Preparations which showed peaks corresponding to molecular weights of less than 14000 on SDS polyacrylamide gel electrophoresis were not used. Type V cytochrome *c* from bovine heart was purchased from Sigma Co.; its concentration was determined using the difference molar extinction coefficient, $19\text{ mM}^{-1}\cdot\text{cm}^{-1}$, between enzymatically reduced and oxidized cytochrome *c* [15]. NADPH was obtained from Oriental Yeast Co., Ltd, Japan. Other reagents were of analytical grade. Spectrophotometric measurements were carried out with a Shimadzu MPS 2000 or Beckman DU 65 spectrophotometer, equipped with a thermostated cell holder, using a kinetic program with a 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.

3. RESULTS AND DISCUSSION

3.1. Rate enhancement of adrenodoxin reduction by adrenodoxin reductase with NADPH in the presence of pyrophosphate or phosphate

The time course of the reduction of adrenodoxin by

Correspondence address: T. Yamano, Laboratory of Chemistry, Kansai Medical University, 18-19 Uyahigashi, Hirakata 573, Japan

the reductase was followed as the decrease in the absorbance at 414 nm at much lower concentrations than equimolar reductase and at a lower temperature, because of the high reaction rates. In the presence of increasing pyrophosphate concentrations, remarkable decreases in absorbance were observed, as shown in Fig. 1. Phosphate ions also caused rate enhancement, but to a lesser extent than pyrophosphate, as can be seen in Fig. 2.

While the rate enhancement by inorganic (pyro)phosphate of adrenodoxin reduction by the reductase and NADPH was remarkable, the rate of reduction of ferricyanide by the reductase with NADPH was similar in Tris-HCl, phosphate and pyrophosphate at pH 7.4 (data not shown).

3.2. Pyrophosphate and phosphate inhibit the reduction of cytochrome *c* via the electron transport system from NADPH to adrenodoxin

Contrary to the enhancement of the rate of reduction of adrenodoxin, inorganic phosphate and pyrophosphate inhibited the electron transfer from adrenodoxin to cytochrome *c*, depending on the (pyro)phosphate concentration. The extent of rate inhibition of cytochrome *c* reduction was independent of the cytochrome *c* concentration. Lineweaver-Burke plots showed that the inhibition by pyrophosphate of the electron transfer to cytochrome *c* was of a non-competitive type. Least squares linear plots in the absence and presence of pyrophosphate gave a K_i value of 9.6 mM. The electron transfer from adrenodoxin to cytochrome *c* was presumably inhibited in such a way that the rate constant of the reaction between them was decreased, leading to apparent non-competitive inhibition.

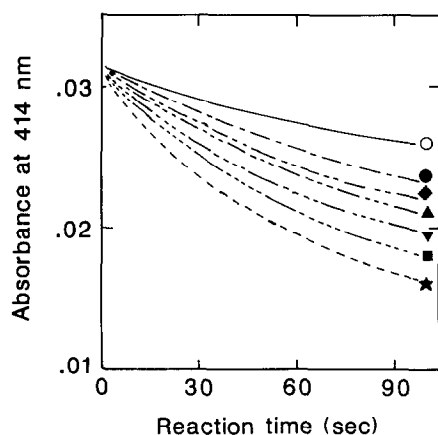


Fig. 1. Time course of the rate enhancement by pyrophosphate of the reduction of adrenodoxin with the reductase and NADPH. Reactions were started by the simultaneous addition of adrenodoxin reductase and NADPH. The temperature was kept at 20°C. The concentrations, in a total volume of 1.0 ml, were: adrenodoxin, 2.8 μ M; adrenodoxin reductase, 6 nM; NADPH, 10 μ M; Tris-HCl, pH 7.4, 25 mM. The pyrophosphate concentrations were: (○) 0 mM; (●) 0.25 mM; (◆) 0.5 mM; (▲) 1.25 mM; (▼) 2.5 mM; (■) 5 mM; (★) 10 mM.

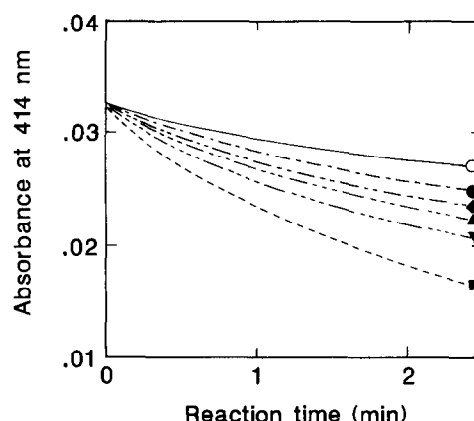


Fig. 2. Time course of the rate enhancement by phosphate of the reduction of adrenodoxin with the reductase and NADPH. The reaction conditions were the same as in Fig. 1, except that pyrophosphate was replaced by phosphate and the phosphate concentrations were: (○) 0 mM; (●) 1.25 mM; (◆) 2.5 mM; (▲) 5 mM; (▼) 10 mM; (■) 50 mM.

tion, but the rate limiting step was still the reaction between adrenodoxin and the reductase.

3.3. Rate enhancement of both the reduction of adrenodoxin and the reduction of cytochrome *c* by the electron transfer system in the presence of nucleotide phosphates

Rate enhancement of the reduction of adrenodoxin by the reductase with NADPH was observed in the presence of ATP. As shown in Fig. 3, the effect of ATP was less remarkable than that of pyrophosphate, but the concentrations used were much lower. The enhancement became saturated and leveled off at less than 50 μ M. The rate of cytochrome *c* reduction was measured with low concentrations of adrenodoxin and a saturated amount of the reductase, because of the

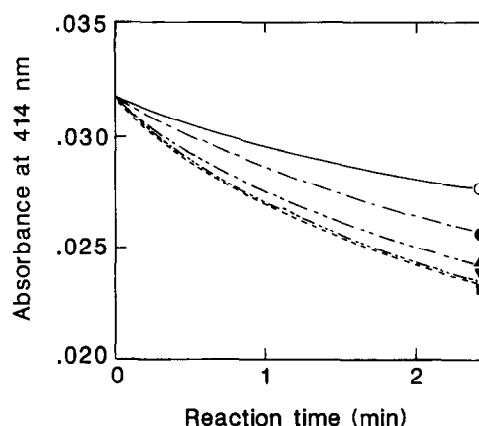


Fig. 3. Time course of the rate enhancement by ATP of the reduction of adrenodoxin with the reductase and NADPH. The reaction conditions were the same as in Fig. 1, except that pyrophosphate was replaced by ATP. The ATP concentrations were: (○) 0 μ M; (●) 5 μ M; (▲) 10 μ M; (▼) 50 μ M; (■) 100 μ M.

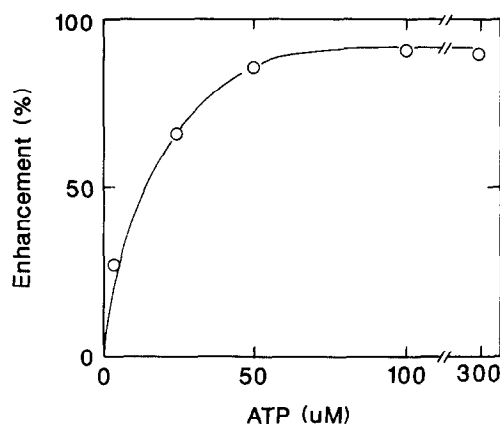


Fig. 4. Percent rate enhancement of the cytochrome *c* reduction with various concentrations of ATP via the electron transfer system from NADPH to adrenodoxin. The reaction temperature was 37°C. The concentrations, in a total volume of 1.0 ml, were: cytochrome *c*, 12 nM; adrenodoxin, 4 nM; adrenodoxin reductase, 60 nM; NADPH, 10 μM; Tris-HCl, pH 7.4, 25 mM.

high rate of reduction of cytochrome *c*. The percent rate enhancement of cytochrome *c* reduction was defined as the ratio of the rate increase in the presence of ATP to the rate in its absence. As shown in Fig. 4, the concentration dependency was hyperbolic. GTP had a similar rate enhancement effect with regard to the extent of enhancement and the concentration dependency. ADP also gave similar results to those in the case of ATP, but AMP was ineffective for the rates of reduction of both adrenodoxin and cytochrome *c*, in contrast to ADP and ATP (data not shown).

Nucleotide phosphates had no appreciable effect on the rate of reduction of ferricyanide by the reductase with NADPH at the concentrations used in these experiments. In anaerobic experiments, it was found that the rate enhancement of cytochrome *c* reduction by ATP is not due to the electron surplus resulting from prevention of the electron flow on autooxidation of reduced adrenodoxin. ATP had no effect as to stabilization of adrenodoxin against heat treatment (data not shown).

Recently, pyrophosphate was shown to accumulate in mitochondria in the presence of Ca^{2+} ions during acetate or butyrate metabolism. The pyrophosphate level reached as high as 30 nmol per mg of mitochondrial protein, which is 10 times the basal level [16].

Hence, there is a possibility that the rate of electron transport is affected by the level of pyrophosphate in mitochondria.

Multiple forms of adrenodoxin have been reported, whose carboxyl terminals are different, presumably due to proteolytic attack during purification [14]. Vickery et al. reported that carboxyl-terminal deleted (des 116–128)-adrenodoxin increased the hydroxylation activity with either P-450_{sc} or P-450_{11β} [17]. Charge interactions between adrenodoxin and ligands may affect the rate of electron transfer at the carboxyl terminal domain.

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