The opposite effect of bivalent cations on cytochrome b_5 reduction by NADH:cytochrome b_5 reductase and NADPH:cytochrome c reductase

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The effects of bivalent cations on cytochrome b_5 reduction by NADH:cytochrome b_5 reductase and NADPH:cytochrome c reductase were studied with the proteinase-solubilized enzymes. Cytochrome b_5 reduction by NADH:cytochrome b_5 reductase was strongly inhibited by CaCl_2 or MgCl_2 . When $1.2\text{ }\mu\text{M}$ -cytochrome b_5 was used, the concentrations of CaCl_2 and MgCl_2 required for 50% inhibition (I_{50}) were 8 and 18 mM respectively. The inhibition was competitive with respect to cytochrome b_5 . The extent of inhibition by CaCl_2 or MgCl_2 was much higher than that by KCl or other alkali halides. In contrast, cytochrome b_5 reduction by NADPH:cytochrome c reductase was extremely activated by CaCl_2 or MgCl_2 . In the presence of 5 mM- CaCl_2 , the activity was 24-fold higher than control when $4.4\text{ }\mu\text{M}$ -cytochrome b_5 was used. The magnitude of activation by CaCl_2 was 2–3-fold higher than that by MgCl_2 . The activation by these salts was much higher than that by KCl, indicating that bivalent cations play an important role in this activation. The mechanisms of inhibition and activation by bivalent cations of cytochrome b_5 reduction by these two microsomal reductases are discussed.

INTRODUCTION

NADH:cytochrome b_5 (cyt. b_5) reductase (EC 1.6.2.2) provides electrons via cyt. b_5 for several kinds of lipid metabolism, such as fatty acid desaturation [1–3], fatty acid elongation [4,5] and cholesterol biosynthesis [6]. This reductase also supports some cytochrome P -450 (cyt. P -450)-mediated drug metabolism by supplying a second electron to cyt. P -450 through cyt. b_5 [7–9].

NADPH:cyt. P -450 reductase (EC 1.6.2.4) is another microsomal electron-transport enzyme, which is involved in a variety of drug metabolism by transferring electrons to cyt. P -450 [7,10,11]. It was also shown that NADPH:cyt. P -450 reductase can reduce cyt. b_5 in a system reconstituted with phospholipid vesicles [12] and is involved in stearyl-CoA desaturation, which is mediated by cyt. b_5 [13].

These facts show that cyt. b_5 can receive electrons from both NADH and NADPH through these two corresponding reductases. We are interested in the mechanism of how these electron-transfer pathways from the two nicotinamide nucleotides to cyt. b_5 are switched or regulated.

NADPH:cytochrome c (cyt. c) reductase is a proteinase (trypsin)-solubilized form of NADPH:cyt. P -450 reductase. It lacks a membrane binding region and cannot reduce cyt. P -450, but reduces cyt. c rapidly [14].

In a previous study [15], we reported that NADH:cyt. b_5 reductase is inhibited by several salts, and the inhibition is caused by the anionic moiety of these salts, such as halides or carboxylates. Univalent cations had little effect on the reaction, but bivalent cations seemed to be inhibitory.

In the present study, we examined the effect of bivalent cations on cyt. b_5 reduction by NADH:cyt. b_5 reductase in more detail, and also their effect on cyt. b_5 reduction by NADPH:cyt. c reductase, because we wanted to know how these cations affect another electron-transport pathway. High ionic strength is known to be necessary for cyt. b_5 reduction by NADPH:cyt. P -450 reductase [12] and also its soluble form, NADPH:cyt. c reductase [16,17].

As a result, we found clearly opposite effects of bivalent cations on cyt. b_5 reduction by these two enzymes, namely that cyt. b_5 reduction by NADH:cyt. b_5 reductase was strikingly inhibited by bivalent cations, whereas that by NADPH:cyt. c reductase was activated remarkably.

MATERIALS AND METHODS

Chemicals

NADH was obtained from Boehringer–Mannheim. NADPH was purchased from Oriental Yeast Co., Tokyo, Japan, and dissolved in 10 mM-glycine/NaOH buffer, pH 10.0, to keep the stock solution alkaline. CaCl_2 and MgCl_2 (both anhydrous) were obtained from Wako Pure Chemicals. All other reagents used are of the highest grade available.

Preparation of cyt. b_5

Trypsin-solubilized cyt. b_5 was purified from bovine or rat liver microsomes (microsomal fractions) by the method of Omura & Takesue [18]. The purified cyt. b_5 was dialysed for 24 h against 5 mM-Tris/HCl buffer, pH 7.0.

Abbreviations used: cyt. b_5 , cytochrome b_5 ; cyt. P -450, cytochrome P -450; cyt. c , cytochrome c ; NADPH:cyt. c reductase, trypsin-solubilized form of NADPH:cyt. P -450 reductase; I_{50} , concentration giving 50% inhibition.

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Fig. 1. Effect of bivalent cations on cyt.*b*₅ reduction by NADH:cyt.*b*₅ reductase

The reaction mixture contained 1.23 μ M bovine cyt.*b*₅, an appropriate amount of NADH:cyt.*b*₅ reductase, and 0.1 mM-NADH in 2 ml of 10 mM-Tris/HCl buffer, pH 7.0. ●, CaCl₂; ○, MgCl₂.

Preparation of NADH:cyt.*b*₅ reductase

Lysosome-solubilized enzyme [19] was purified from bovine liver microsomes as described previously [20,21].

Preparation of NADPH:cyt.*c* reductase

Trypsin-solubilized NADPH:cyt.*c* reductase was purified from rat liver microsomes by the method of Omura & Takesue [18]. Purified enzyme has a specific activity of 24.5 μ mol of cyt.*c* reduced/min per mg in 0.1 M-potassium phosphate buffer, pH 7.5 at 25 °C.

Assay of cyt.*b*₅ reduction

All assays were carried out at 25 °C with a Union SM-401 spectrophotometer. The reaction mixture contained 1.23–4.75 μ M-cyt.*b*₅, 0.1 mM-NADH or -NADPH, and an appropriate amount of enzyme in 2 ml or 0.6 ml of 10 mM-Tris/HCl buffer, pH 7.0, containing 0–0.125 M-CaCl₂ or -MgCl₂. The reaction was started by addition of NADH or NADPH.

Cyt.*b*₅ reduction was measured by monitoring the increase in the A_{424} . The difference absorption coefficient 100 mM⁻¹·cm⁻¹ [22] was used in the calculation.

Protein content was determined by the method of Lowry *et al.* [23] with bovine serum albumin as standard.

RESULTS

Effect of bivalent cations on cyt.*b*₅ reduction by NADH:cyt.*b*₅ reductase

The effects of CaCl₂ and MgCl₂ on cyt.*b*₅ reduction by NADH:cyt.*b*₅ reductase were examined. Fig. 1 shows

Fig. 2. Lineweaver-Burk plots of NADH:cyt.*b*₅ reductase activity in the presence of different concentrations of MgCl₂

Assay conditions were the same as for Fig. 1, except that the concentration of cyt.*b*₅ was varied (0.37–1.23 μ M). The reaction mixture contained 0 (●), 25 mM- (○), 50 mM- (■), 75 mM- (□), 100 mM- (▲) or 125 mM- (△) MgCl₂.

the activities in the presence of these salts at various concentrations; these salts strongly inhibited this cyt.*b*₅ reduction. The extent of inhibition by CaCl₂ was higher than that by MgCl₂. The concentrations of CaCl₂ and MgCl₂ giving 50% inhibition (I_{50}) were 8.1 and 18.1 mM respectively.

To study the kinetics of inhibition, the reaction was performed with different concentrations of cyt.*b*₅ in the presence of CaCl₂ and MgCl₂. Fig. 2 shows the Lineweaver-Burk plots of the results with MgCl₂, indicating that the inhibition by MgCl₂ was competitive with respect to cyt.*b*₅. A similar result was obtained with CaCl₂.

We previously found that Cl⁻ ion inhibits cyt.*b*₅ reduction by NADH:cyt.*b*₅ reductase competitively [15]. Therefore we compared the extent of the inhibition by CaCl₂ or MgCl₂ with that by KCl. Fig. 3 shows apparent K_m values determined in the presence of these salts. The extent of inhibition by CaCl₂ or MgCl₂ was much higher than that expected by Cl⁻ ion. This result indicates that Ca²⁺ and Mg²⁺ play a major role in the inhibition by CaCl₂ and MgCl₂, although the contribution of Cl⁻ ion cannot be eliminated.

The plots of K_m versus [MgCl₂] or [CaCl₂] are not linear but parabolic, indicating that this inhibition is not pure competitive inhibition [24].

Effect on cyt.*b*₅ reduction by NADPH:cyt.*c* reductase

Next, we examined the effects of CaCl₂ and MgCl₂ on

Fig. 3. Effect of bivalent cations on apparent K_m for cyt. b_5 of NADH:cyt. b_5 reductase

Assay conditions were the same as for Fig. 2. ○, CaCl_2 ; □, KCl; ●, MgCl_2 .

Fig. 4. Effect of CaCl_2 on cyt. b_5 reduction by NADPH:cyt. c reductase

The reaction mixture contained $4.75 \mu\text{M}$ rat cyt. b_5 , $2 \mu\text{l}$ of NADPH:cyt. c reductase and 0.12 mM -NADPH, in 10 mM -Tris/HCl buffer, pH 7.4 (0.6 ml). Other experimental conditions were the same as described in the Materials and methods section.

Fig. 5. Effect of bivalent cations on cyt. b_5 reduction by NADPH:cyt. c reductase

The reaction mixture contained $1.43 \mu\text{M}$ rat cyt. b_5 , $5 \mu\text{l}$ of NADPH:cyt. c reductase and 0.1 mM -NADPH in 2 ml of 10 mM -Tris/HCl, pH 7.4. ●, CaCl_2 ; ○, MgCl_2 .

cyt. b_5 reduction by NADPH:cyt. c reductase. Fig. 4 shows the effect of CaCl_2 on cyt. b_5 reduction when $4.8 \mu\text{M}$ -cyt. b_5 was used as an acceptor. In the absence of Ca^{2+} the activity was very low. The activity was increased by increasing Ca^{2+} concentrations. With 1 mM -, 2 mM - and 5 mM - CaCl_2 , the activity was 4-, 9- and 22-fold higher than control respectively.

Fig. 5 shows the concentration-dependence of the activation on CaCl_2 or MgCl_2 . The extent of activation by CaCl_2 was 2–3-fold higher than that by MgCl_2 in the range of 0 – 150 mM . The activity was increased with increasing CaCl_2 concentrations up to 100 mM , and at higher concentrations it was gradually decreased. For MgCl_2 , a broad peak of activation was found at around 200 mM (results not shown).

To find whether the activation was caused by ionic strength or the cation or anion moiety of these salts, we examined the effect of several other salts. Table 1 summarizes the activities in the presence of CaCl_2 , MgCl_2 and other salts when $1.4 \mu\text{M}$ -cyt. b_5 was used as an electron acceptor. The activities with CaCl_2 and MgCl_2 were apparently higher than with KCl or other salts at 10 mM . These results indicated that the activation by CaCl_2 or MgCl_2 is caused by the effect of bivalent cations and is not explained by an increase in ionic strength.

To get more information on the mechanism of activation, we determined apparent K_m and $V_{\max}$ values at different concentrations of CaCl_2 (Table 2). The K_m value for cyt. b_5 was decreased by addition of CaCl_2 . It was $33.3 \mu\text{M}$ in the absence of CaCl_2 , and in the presence of 1 mM - and 50 mM - CaCl_2 it was $16.1 \mu\text{M}$ and $11.1 \mu\text{M}$

Table 1. Effect of various salts on cyt.*b*₅ reduction by NADPH:cyt.*c* reductase

The assay mixture contained 1.4 μ M-cyt.*b*₅, 0.1 mM-NADH or -NADPH and 10 μ l of enzyme preparation (2.2 mg/ml) in 2 ml of 10 mM-Tris/HCl buffer (pH 7.5) containing the salts indicated. The values in parentheses are the activities at 100 mM salt concentration.

Salt (10 mM)	Cyt. <i>b</i> ₅ reduction (nmol/min per mg)
Control	8
CaCl ₂	155
MgCl ₂	64
KCl	9 (12)
(NH ₄) ₂ SO ₄	13 (46)
Potassium phosphate buffer, pH 7.5	10 (21)

Table 2. Effect of CaCl₂ on *K*_m for cyt.*b*₅ of NADPH:cyt.*c* reductase

Apparent *K*_m values were determined from Lineweaver-Burk plots of the reactions with 1.4 μ M-, 2.0 μ M-, 2.9 μ M- and 5.7 μ M-cyt.*b*₅.

Activator	Apparent <i>K</i> _m for cyt. <i>b</i> ₅ (μ M)	<i>V</i> _{max.} (μ mol/min per mg)
Control	33.3	0.07
1 mM-CaCl ₂	16.1	0.16
50 mM-CaCl ₂	11.1	7.7

respectively. On the other hand, *V*_{max.} was increased greatly by addition of CaCl₂. The values at 1 mM- and 50 mM-CaCl₂ were 2- and 100-fold higher than the control respectively.

DISCUSSION

Recent studies [7–13] on microsomal electron-transport systems have showed that cyt.*b*₅ plays an important role in connecting two electron-transfer pathways from NADH and NADPH through NADH:cyt.*b*₅ reductase and NADPH:cyt.*P*-450 reductase respectively.

In the present study, we investigated the effect of bivalent cations on cyt.*b*₅ reduction by these two microsomal reductases. To simplify the reaction system, we used here proteinase-solubilized enzymes. NADH:cyt.*b*₅ reductase was solubilized by lysosomal digestion [19,20], and NADPH:cyt.*P*-450 reductase was solubilized by trypsin [18]. Structural studies of these enzymes showed that both proteinase-solubilized enzymes lack the *N*-terminal membrane-binding region [21,25,26].

Inhibition of cyt.*b*₅ reduction by NADH:cyt.*b*₅ reductase

Bivalent cations gave a remarkable inhibition of cyt.*b*₅ reduction by NADH:cyt.*b*₅ reductase. The extent of inhibition was higher than would be expected from the chloride concentration (Fig. 3), which is inhibitory to

this reaction [15]. Thus Ca²⁺ or Mg²⁺ itself seemed to be inhibitory to the reaction. The kinetics of inhibition was found to be competitive with respect to cyt.*b*₅, and the plots of *K*_m versus [salt] were not linear, but parabolic (Fig. 3). These features are similar to those observed with alkali or ammonium halides. However, the mechanism of inhibition by CaCl₂ or MgCl₂ may be more complicated, because in this case both anion and cation seemed inhibitory.

Cyt.*b*₅ is an acidic protein, and has an 'anionic site' on its surface, consisting of five carboxy groups and haem propionate [27,28]. Dailey *et al.* [28] proposed that NADH:cyt.*b*₅ reductase interacts with cyt.*b*₅ by forming complementary charge-pairing between amino groups of the reductase and the 'anionic site' of cyt.*b*₅.

In our previous paper [15], we speculated that inhibition by anions is caused by the binding of anions to amino groups in the cyt.*b*₅-binding site of the reductase. In the present study, we assumed that bivalent cations bind to the 'anionic site' of cyt.*b*₅, because bivalent cations have high affinity to two or more adjacent carboxy groups. We considered that the strong inhibition by CaCl₂ or MgCl₂ is caused by binding of bivalent cations to cyt.*b*₅ and of Cl[−] ions to NADH:cyt.*b*₅ reductase.

Activation of cyt.*b*₅ reduction by NADPH:cyt.*c* reductase

Several workers reported that cyt.*b*₅ reduction by NADPH:cyt.*c* (*P*-450) reductase is activated by high concentrations of salts such as (NH₄)₂SO₄ [16], KCl [17] and phosphate buffer [28,29]. The salt concentrations used in those studies were high (0.3–2 M), and the effect was assumed to be an ionic-strength effect.

In the present study, we found that CaCl₂ or MgCl₂ activated the reaction at lower concentration. Comparison with the results with other salts (Table 1) showed that the effect is not an ionic-strength effect, but a specific effect of bivalent cations. The kinetic study showed that these bivalent cations decreased *K*_m for cyt.*b*₅ and increased *V*_{max.} greatly (Table 2).

How does the activation by bivalent cations occur? The mechanism by activation is not clear at present, but the decrease in *K*_m for cyt.*b*₅ could be due to the effect on enzyme-substrate (protein-protein) interaction.

The nature of the cyt.*b*₅-binding site of NADPH:cyt.*P*-450 reductase is thought to be cationic [13,30,31] and different from the cyt.*P*-450-binding site [31]. Dailey & Strittmatter [13] proposed that haem propionate and other carboxy groups on the 'anionic site' of cyt.*b*₅ may interact with amino groups of NADPH:cyt.*P*-450 reductase. However, more recently Tamburini and co-workers showed that blocking of haem propionate [29] or carboxy groups [32] of cyt.*b*₅ resulted in a great stimulation of electron transfer from NADPH:cyt.*c* reductase to cyt.*b*₅. It was suggested that the negative charge repulsion between the 'anionic site' of cyt.*b*₅ and negative charges of the reductase may impair the interaction between these proteins, and the removal of negative charge may result in stimulation [29].

In the present system, it is likely that bivalent cations bind to carboxy groups of the 'anionic site' of cyt.*b*₅, and also to negative charges of the reductase, to eliminate the electrostatic repulsion. The decrease in the *K*_m value for cyt.*b*₅ may support this possibility.

It is also possible that bivalent cations may affect other step(s) than protein-protein interaction. The increase in $V_{\max}$ by bivalent cations (Table 2) seems to suggest this possibility. Further study is required to clarify the mechanism of this activation.

In this study, Ca^{2+} was more effective in both inhibition of NADH:cyt. b_5 reductase and activation of NADPH:cyt. c reductase than was MgCl_2 . However, the effect of Mg^{2+} cannot be ignored, because the concentration of Mg^{2+} in cells is higher than that of Ca^{2+} [33].

A previous study by Hildebrandt & Estabrook [7] showed that mixed-function oxidation of ethylmorphine, which is thought to be cyt. b_5 -dependent, was stimulated by Mg^{2+} at around 5 mM. This observation seems interesting in considering the physiological meaning of the bivalent-cation effect.

In this study, cyt. b_5 reduction by NADPH:cyt. c reductase was shown to be greatly increased by bivalent cations. The rate of reduction, however, was still much lower than that with NADH:cyt. b_5 reductase, although we cannot compare exactly, because we purified these enzymes from different species. It was shown that the presence of the membrane-binding segment accelerates the reduction rate [12]. We suppose that in the presence of bivalent cations the reduction rate by intact enzyme is also higher than that by proteinase-solubilized enzyme. The study using detergent-solubilized enzyme or microsomes in the presence of bivalent cations should be expected.

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REFERENCES

- Holloway, P. W. & Wakyl, S. J. (1970) *J. Biol. Chem.* **245**, 1862–1865
- Strittmatter, P., Spatz, L., Corcoran, D., Rogers, K. J., Setlow, B. & Redline, R. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4565–4569
- Okayasu, T., Ono, T., Shinogima, K. & Imai, Y. (1977) *Lipids* **12**, 267–271
- Keyes, S. R. & Cinti, D. L. (1980) *J. Biol. Chem.* **255**, 11357–11364
- Takeshita, M., Tamura, M., Yoshida, S. & Yubisui, T. (1985) *J. Neurochem.* **45**, 1390–1395
- Reddy, V. V. R., Kupfer, D. & Capsi, E. (1977) *J. Biol. Chem.* **252**, 2797–2801
- Hildebrandt, A. & Estabrook, R. W. (1971) *Arch. Biochem. Biophys.* **143**, 66–79
- Omura, T. (1978) in *Cytochrome P-450* (Sato, R. & Omura, T., eds.), pp. 138–163, Academic Press, New York
- Oshino, N. (1982) in *Hepatic Cytochrome P-450 Monooxygenase System* (Schenkman, J. B. & Kupfer, D., eds.), pp. 424–447, Pergamon Press, Oxford and New York
- Lu, A. Y. H., Levine, W., Selander, H. & Jerina, D. M. (1974) *Biochem. Biophys. Res. Commun.* **61**, 1348–1355
- Sugiyama, T., Miki, N. & Yamano, T. (1979) *Biochem. Biophys. Res. Commun.* **90**, 715–720
- Enoch, H. G. & Strittmatter, P. (1979) *J. Biol. Chem.* **254**, 8976–8981
- Dailey, H. A. & Strittmatter, P. (1980) *J. Biol. Chem.* **255**, 5184–5189
- Phillips, A. H. & Langdon, R. G. (1962) *J. Biol. Chem.* **237**, 2652–2660
- Tamura, M., Yubisui, T. & Takeshita, M. (1985) *Biochem. J.* **230**, 273–276
- Bilimoria, M. H. & Kamin, H. (1973) *Ann. N.Y. Acad. Sci.* **212**, 428–448
- Prough, R. A. & Masters, B. S. S. (1974) *Arch. Biochem. Biophys.* **165**, 263–267
- Omura, T. & Takesue, S. (1970) *J. Biochem. (Tokyo)* **67**, 249–257
- Takesue, S. & Omura, T. (1970) *J. Biochem. (Tokyo)* **67**, 267–276
- Tamura, M., Yubisui, T. & Takeshita, M. (1983) *J. Biochem. (Tokyo)* **94**, 1547–1555
- Tamura, M., Yubisui, T., Takeshita, M., Kawabata, S., Miyata, T. & Iwanaga, S. (1987) *J. Biochem. (Tokyo)* **101**, 1147–1159
- Strittmatter, P. & Velick, S. F. (1957) *J. Biol. Chem.* **228**, 785–799
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Segel, I. H. (1975) *Enzyme Kinetics*, pp. 465–470, John Wiley and Sons, New York
- Black, S. D. & Coon, M. J. (1982) *J. Biol. Chem.* **257**, 5929–5938
- Haniu, M., Iyanagi, T., Legesse, K. & Shively, J. E. (1984) *J. Biol. Chem.* **259**, 13703–13711
- Mathews, F. S. (1976) in *The Enzymes of Biological Membranes* (Martonosi, A., ed.), vol. 4, pp. 143–198, Plenum Press, New York
- Dailey, H. A. & Strittmatter, P. (1979) *J. Biol. Chem.* **254**, 5388–5396
- Tamburini, P. P. & Schenkman, J. B. (1986) *Arch. Biochem. Biophys.* **245**, 512–522
- Nisimoto, Y. & Lambeth, J. D. (1985) *Arch. Biochem. Biophys.* **241**, 386–396
- Tamburini, P. P., MacFarquhar, S. & Schenkman, J. B. (1986) *Biochem. Biophys. Res. Commun.* **134**, 519–526
- Tamburini, P. P., White, R. E. & Schenkman, J. B. (1985) *J. Biol. Chem.* **260**, 4007–4015
- Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. & Watson, J. D. (1983) *Molecular Biology of the Cell*, p. 286, Garland Publishing, New York

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