The inhibitory effect of halides and carboxylates on hepatic NADH:cytochrome b_5 oxidoreductase

Minoru TAMURA, Toshitsugu YUBISUI and Masazumi TAKESHITA
Department of Biochemistry, Medical College of Oita, Hazama-cho, Oita, 879-56, Japan

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The inhibitory effect of several halides and carboxylates on bovine liver microsomal NADH:cytochrome b_5 oxidoreductase (EC 1.6.2.2) was examined. The magnitude of inhibition was altered by various anion species but not by cation species. The order of effectiveness was $F^- < \text{acetate} < Cl^- < Br^- < I^- < \text{succinate} \approx \text{citrate}$. The kinetics of these inhibitions was competitive with cytochrome b_5 , and non-competitive with NADH, indicating that these anions inhibit the interaction of the enzyme with cytochrome b_5 .

Inhibition of cytochrome b_5 reductase (NADH:cytochrome b_5 oxidoreductase, EC 1.6.2.2) activity by several salts or buffers has been reported with the enzymes of erythrocytes (Passon & Hultquist, 1972; Yubisui & Takeshita, 1982), of liver microsomal fractions (Strittmatter & Velick, 1957; Iyanagi *et al.*, 1984) and of brain microsomal fractions (Tamura *et al.*, 1983). Strittmatter & Velick (1957) had early reported that cytochrome b_5 reductase of calf liver was inhibited by some chelating agents, such as pyrophosphate and EDTA. Passon & Hultquist (1972) reported that the enzyme of human erythrocytes was inhibited by $Tris^+$ or K^+ . It was also reported that the erythrocyte enzyme has an unusual pH-activity profile when different buffers were used in the assay (Passon & Hultquist, 1972; Goto-Tamura *et al.*, 1976).

In spite of these observations, the mechanism of the inhibition by salts has not yet been elucidated. It was uncertain whether the inhibition is caused by an ionic-strength effect (Debye-Hückel theory) or by specific binding of cation or anion species.

In a previous paper (Tamura *et al.*, 1983) we reported that the purified brain enzyme was inhibited by phosphate buffer or by several salts competitively.

In the present study, to clarify the mechanism of the salt inhibition, we studied the effects of several alkali (ammonium) halides and carboxylates on cytochrome b_5 reductase activity in detail. On the basis of the results obtained, the mechanism of the inhibition is discussed.

Materials and methods

Chemicals

All chemicals used were of analytical-reagent grade.

Purification of cytochrome b_5

Trypsin-solubilized cytochrome b_5 was purified from bovine liver microsomal fraction by the method of Omura & Takesue (1970). The purified cytochrome b_5 was dialysed for 24 h against 1 mM-potassium phosphate buffer, pH 7.0.

Purification of bovine liver microsomal cytochrome b_5 reductase

The enzyme was purified as described by Tamura *et al.* (1983) except for the following two points. The liver was homogenized with 0.15 M-KCl/1 mM-EDTA, pH 7.5. Also, the active fractions eluted from 5'-AMP-Sepharose column were further purified with a Sephadex G-75 column equilibrated with 10 mM-potassium phosphate buffer, pH 7.5, containing 1 mM-EDTA and 0.2 mM-dithiothreitol. The purified enzyme showed one band, of M_r 31 000, on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969). The purified enzyme could be stored at 0°C at least for a month without any detectable loss of activity.

Assay system

All assays were carried out at 25°C with a Union SM-401 spectrophotometer. Unless otherwise stat-

ed, cytochrome b_5 reductase activity was measured by monitoring the increase of the absorbance at 424 nm; $100\text{ mm}^{-1}\cdot\text{cm}^{-1}$ was used as the difference millimolar absorption coefficient between reduced and oxidized cytochrome b_5 (Strittmatter & Velick, 1957). The reaction mixture contained 0.37–3.43 μM -cytochrome b_5 , 2.2 nM purified enzyme and 0.1 mM-NADH in 2 ml of 1 mM-potassium phosphate buffer, pH 7.0, containing 0–0.25 M-salt or in 2 ml of 0.05–0.25 M-sodium acetate, -sodium succinate or -sodium citrate buffer, pH 7.0. The solutions of organic acids and ammonium chloride derivatives (NH_4Cl , Tris chloride and guanidinium chloride) were neutralized to pH 7.0 with 0.02–0.1 M-NaOH for the experiments. Apparent K_m values for substrates were determined from Lineweaver-Burk plots of the results with different concentrations of a substrate.

Protein content was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. The concentration of the enzyme was determined spectrophotometrically, on the basis of a millimolar absorption coefficient of $10.2\text{ mm}^{-1}\cdot\text{cm}^{-1}$ at 460 nm (Strittmatter & Velick, 1957).

Results

Effect of alkali halides

Inhibitions by several alkali halides were examined. The kinetics of the inhibitions by alkali halides tested were all competitive with respect to cytochrome b_5 . Table 1 summarizes the apparent K_m values in the presence of several alkali halides at various concentrations. When the anion species was varied, the extent of inhibition was apparently altered and the effectiveness in inhibition increased in the order $\text{F}^- < \text{Cl}^- < \text{Br}^- < \text{I}^-$ (Fig. 1). In contrast, it was practically unaltered by varying the cation species in chlorides. These

results suggest that the effective inhibitor species is the anion.

An attempt to estimate the K_i values from Dixon plots ($1/v$ versus $[\text{I}]$) was unsuccessful because of non-linearity of the plots. The plots of K_m versus $[\text{I}]$ were not linear, but parabolic, as shown in Fig. 1.

Effect of ammonium chloride derivatives

The effect of several compounds such as NH_4Cl , Tris chloride and guanidinium chloride was exam-

Fig. 1. Effect of various sodium halides on apparent K_m for cytochrome b_5 . Alkali halides used were NaF (F^-), NaCl (Cl^-), NaBr (Br^-) and NaI (I^-). Assay conditions were as described in the Materials and methods section.

Table 1. Effect of several halides on apparent K_m for cytochrome b_5 of cytochrome b_5 reductase activity. The assay conditions were as described in the Materials and methods section. The K_m value measured in the absence of alkali halides was 3.1 μM . V_{max} of these reactions was 1520 units/mg.

Alkali halide	Concn.	Apparent K_m for cytochrome b_5 (mM)				
		0.05M	0.10M	0.15M	0.20M	0.25M
NaF		5.1	9.2	13.8	18.5	25.4
NaCl		6.1	12.1	20.4	28.1	40.5
NaBr		9.2	22.3	39.6	62.6	90.5
NaI		11.5	30.2	55.2	87.4	134.6
LiCl		7.0	12.6	19.4	29.4	42.5
KCl		6.9	13.4	19.7	30.6	44.8
RbCl		7.3	12.2	19.2	28.3	41.2
NH_4Cl		8.7	14.4	22.0	31.5	45.8
Tris chloride		6.7	12.2	20.6	30.1	45.0
Guanidinium chloride		6.7	11.0	18.6	28.1	41.3

Table 2. *Effect of NaCl and sodium succinate on K_m for NADH and V_{max} of cytochrome b_5 reductase activity*
The reaction mixture contained 2.02 nM-enzyme, 0.3–3 μ M-NADH and 28.3 μ M-cytochrome b_5 . The assay was carried out by monitoring the increase in the absorbance at 556 nm and using a millimolar absorption coefficient of 19 mm $^{-1}$ ·cm $^{-1}$ (Omura & Takesue, 1970).

Salt	K_m (NADH) (μ M)	V_{max} . (units/mg)
None	0.91	1420
0.25 M-NaCl	0.86	578
0.25 M-Sodium succinate	0.99	473

Fig. 2. *Effect of carboxylates on apparent K_m for cytochrome b_5*

Assay conditions were as described in the Materials and methods section.

ined. The extent of inhibition by these salts was nearly equal to or similar to that by alkali chlorides (Table 1). This result confirms that anions are mainly responsible for the inhibition.

Effect of carboxylates

The effect of acetate, succinate and citrate was examined. The inhibitions by these salts were also competitive with respect to cytochrome b_5 . Fig. 2 shows the plots of apparent K_m against the concentration of these carboxylates. The extent of inhibition by acetate was intermediate between those by Cl^- and F^- . Inhibitions by succinate and citrate were almost identical, and much higher than that with acetate. It was noteworthy that plots of K_m for cytochrome b_5 versus [salt] with multivalent carboxylates seemed to be closer to a straight line than those with univalent anions. Dixon plots of these multivalent carboxylates were

almost linear, and the K_i values were estimated to be 8–10 mM.

Effect of anions on NADH binding

The effect of anions on the interaction of the enzyme with NADH was examined. Table 2 summarizes values of K_m for NADH and V_{max} in the absence and in the presence of NaCl or sodium succinate when the concentration of the cytochrome b_5 was 28.3 μ M. K_m for NADH was not altered by increasing the concentration of these salts, although V_{max} was decreased with an increase of the concentration of these salts. These results indicate that these salts did not inhibit the binding of NADH with the enzyme.

Reversibility of inhibition

The reversibility of inhibition was examined by dilution experiments. The activity in the presence of 0.25 M-NaBr was 4.2% of control. The activity was restored to 36.4% by 5-fold dilution, and this activity is almost identical with that with 0.05 M-NaBr. Similar results were obtained with other alkali halides and carboxylates used in this work. The results indicated that the inhibition was reversible.

Discussion

The extent of inhibition by univalent anions was in the order $\text{F}^- < \text{acetate} < \text{Cl}^- < \text{Br}^- < \text{I}^-$. This order is in accord with a Hofmeister series, and interestingly it is also consistent with the selectivity coefficient of anion species to anion-exchange resins with quaternary ammonium groups (Wheaton & Bauman, 1951). Therefore it is considered that the order of inhibition may reflect the affinity of each anion for a cationic group.

A carboxy group can be assumed as a model group for the residues on cytochrome b_5 that are thought to be involved in charge-pair interaction (Dailey & Strittmatter, 1979). The inhibition by acetate was relatively low among anions tested. However, succinate was a stronger inhibitor than acetate. The inhibition by citrate was similar to that by succinate. These results suggest that two carboxy groups in a molecule of succinate or

citrate could bind simultaneously to anion-binding sites at the active centre on the enzyme.

The reaction mechanism for cytochrome b_5 reductase is considered to be ordered Bi Bi (Strittmatter, 1966; Iyanagi, 1977; Abe & Sugita, 1979; Yubisui & Takeshita, 1982). The kinetics of the inhibition was competitive with respect to cytochrome b_5 and non-competitive with respect to NADH when cytochrome b_5 is not saturated. This indicated that the inhibition occurred in the interaction of the enzyme with cytochrome b_5 , not NADH. At the same time, it suggested that anions bind to both E and E-NADH complex because, if anions bind only to E-NADH complex, the inhibition should be uncompetitive with respect to NADH. Also, when ferricyanide was used in large excess as an electron acceptor, both K_m for NADH ($2.2\ \mu\text{M}$) and V_{max} were unchanged by the concentration of these salts (M. Tamura, T. Yubisui & M. Takeshita, unpublished work).

A plot of K_m for cytochrome b_5 against $[I]$ was not linear but parabolic. This parabolic competitive inhibition can be explained by multiple binding of inhibitors (anions) to the active site of the enzyme (Segel, 1975). This idea is supported by the fact that the plots with multivalent carboxylates were closer to a straight line than were those with univalent anions.

How many anions can bind to the active site of the enzyme is not clear. However, there may be two or more anion-binding sites having the higher affinity for anions at the active centre. Loverde & Strittmatter (1968) reported that modification of 0.9 and 2.5 lysine residues per enzyme molecule by trinitrophenylation resulted in 27% and 16% respectively of the activity of native enzyme.

On the basis of the results described above, we conclude that the inhibition by alkali halides and carboxylates was caused by the binding of anion species to anion-binding sites at the cytochrome b_5 site on the reductase.

Although univalent cations were found not to be inhibitory in this work, bivalent cations such as Mg^{2+} and Ca^{2+} were apparently inhibitory (M.

Tamura, T. Yubisui & M. Takeshita, unpublished work). In addition, inorganic multivalent anions such as phosphate and sulphate also inhibited more strongly than Br^- did. The details of the inhibition by these multivalent ions and their mechanisms require further investigation.

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References

- Abe, K. & Sugita, Y. (1979) *Eur. J. Biochem.* **101**, 423–428
- Dailey, H. A. & Strittmatter, P. (1979) *J. Biol. Chem.* **254**, 5388–5396
- Goto-Tamura, R., Takesue, Y. & Takesue, S. (1976) *Biochim. Biophys. Acta* **423**, 293–302
- Iyanagi, T. (1977) *Biochemistry* **16**, 2725–2730
- Iyanagi, T., Watanabe, S., & Anan, F. K. (1984) *Biochemistry* **23**, 1418–1425
- Loverde, A. & Strittmatter, P. (1968) *J. Biol. Chem.* **243**, 5779–5787
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Omura, T. & Takesue, S. (1970) *J. Biochem. (Tokyo)* **67**, 249–257
- Passon, P. G. & Hultquist, D. E. (1972) *Biochim. Biophys. Acta* **275**, 62–73
- Segel, I. H. (1975) *Enzyme Kinetics*, pp. 465–470, John Wiley and Sons, New York
- Strittmatter, P. (1966) in *Flavin and Flavoproteins* (Slater, E. C., ed.), pp. 306–340, Elsevier, Amsterdam
- Strittmatter, P. & Velick, S. F. (1957) *J. Biol. Chem.* **228**, 785–799
- Tamura, M., Yubisui, T. & Takeshita, M. (1983) *J. Biochem. (Tokyo)* **94**, 1547–1555
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Wheaton, R. M. & Bauman, W. C. (1951) *Ind. Eng. Chem.* **43**, 1088–1093
- Yubisui, T. & Takeshita, M. (1982) *J. Biochem. (Tokyo)* **91**, 1467–1477