Biochimica et Biophysica Acta, 616 (1980) 22—29

BBA 69124

HUMAN ERYTHROCYTE NADH: (ACCEPTOR) OXIDOREDUCTASE

KINETIC PROPERTIES AND COMPETITIVE SUBSTRATE INHIBITION BY FERRICYANIDE

CHI-SUN WANG

The Laboratory of Lipid and Lipoprotein Studies, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104 (U.S.A.)

(Received April 21st, 1980)

Key words: NADH dehydrogenase; Competitive substrate inhibition; Kinetic mechanism; Ferricyanide; (Human erythrocyte)

Summary

The kinetic mechanism of human erythrocyte membrane NADH: (acceptor) oxidoreductase (EC 1.6.99.3) was studied by product and substrate inhibition kinetics. With ferricyanide as the substrate, the enzyme reaction follows an ordered bi-bi reaction mechanism. The Michaelis-Menten constants for NADH and ferricyanide are 0.014 and 0.049 mM, respectively. The competitive substrate inhibition effect of ferricyanide was due to its direct binding to the enzyme and forming an inactive binary complex. The dissociation constant of the complex was determined to be 0.37 mM.

Introduction

Zamudio and co-workers [1] have demonstrated that human red cells contain a membrane-bound NADH: (acceptor) oxidoreductase (EC 1.6.99.3) which is capable of oxidizing NADH in the presence of electron acceptors such as ferricyanide, cytochrome *c* and 2,6-dichlorophenolindophenol. The enzyme was not released from the membrane by sonication, freeze-thaw [1]or by EDTA treatment [2]. Therefore, it can be considered that NADH: (acceptor) oxidoreductase is an integral membrane protein [1]. We have already confirmed the presence of the enzyme in the human erythrocyte membrane and devised a procedure for its purification by isoelectric focusing and NAD^+-Sepharose 4B affinity chromatography. The study [2] indicated that the enzyme is a glyco-

protein with a subunit molecular weight of 40 000. It was also suggested that the enzyme might be a transmembrane protein with the catalytic site localized at the inner surface and the carbohydrate moiety at the external surface of the membrane. The purified enzyme had a specific activity of 112 μmol/min per mg protein: a turnover number of 4480 min^{-1} for the subunit of 40 000 daltons. In this paper, I describe the kinetic mechanism of the purified enzyme using product and substrate inhibition kinetics.

Experimental procedures

Materials. Unless otherwise stated, all chemicals were purchased from Sigma Chemical Company, St. Louis, MO. The NADH: (acceptor) oxidoreductase was purified from human erythrocyte ghosts as described previously [2]. The enzyme was pure as tested by SDS-polyacrylamide gel electrophoresis and immunochemical criteria.

Enzyme assay. The rates of reduction of ferricyanide were determined in a Beckman model 25 spectrophotometer with a recorder and temperature control unit. The enzyme reactions were performed at 37°C in the presence of 50 mM Tris-HCl buffer, pH 7.5/0.1% Triton X-100 in a final volume of 3 ml. NADH and potassium ferricyanide concentrations were as described in the figure legends. Initial reaction rates were determined from the linear portion of the absorbance change at 420 nm. The mmol extinction coefficients used were NADH, 6.22 (340 nm) and potassium ferricyanide, 0.96 (420 nm). Protein was determined as described previously [3].

Analysis of results. The linearity of all plots was checked graphically and the data were analyzed by least-squares linear regression, assuming a constant variance of reaction velocities (with a Compucorp. 344 Micro Statistician Computer). The measured initial rates with lowest substrate concentrations had coefficients of variation less than 10%.

Results and Discussion

Elucidation of reaction mechanism of NADH: (acceptor) oxidoreductase catalyzed reaction

The enzymic reaction has an absolute requirement for NADH as the cofactor; NADPH is not effective for ferricyanide reduction. The standard mid point potentials for ferrocyanide/ferricyanide and NADH + H^+/NAD^+ are 0.36 V and —0.32 V, respectively. The high oxidation-reduction potential of the system explains the observed irreversibility of the enzyme catalyzed reaction. Therefore, only the forward reaction was examined in studying the reaction mechanism. It was observed that NAD^+ was a competitive product inhibitor with NADH as the variable substrate (Fig. 1) and a non-competitive product inhibitor with ferricyanide as the variable substrate (Table I). On the other hand, ferrocyanide showed non-competitive inhibition both with NADH (Table I) and ferricyanide (Fig. 2) as variable substrates. A summary of product inhibition patterns is shown in Table I.

The results from product inhibition studies comply with an ordered bi-bi reaction mechanism as described by Cleland [4]. For such a mechanism com-

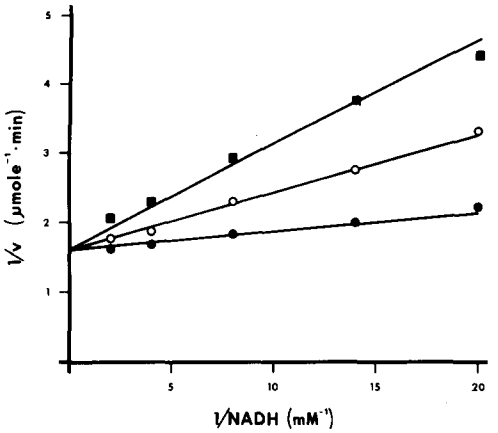

Fig. 1. Competitive product inhibition effect of NAD^+ on NADH : ferricyanide oxidoreductase activity. The concentrations of NAD^+ were: ○ (●———●), 2 mM (○———○) and 5 mM (■———■). Concentration of ferricyanide was 0.33 mM; enzyme concentration 2 μg/ml.

petitive product inhibition is expected only for the product and substrate that interact with the free enzyme, i.e., only one of four possible substrate and product pairs for this enzyme. This was indeed the case since NAD^+ behaved as the only competitive product inhibitor with NADH as the variable substrate (Table I). For a rapid-equilibrium bi-bi mechanism or ping-ping bi-bi mechanism, additional competitive inhibition would be expected with ferrocyanide as a product inhibitor. Also, in a special case of ordered bi-bi mechanism where enzyme-substrate central ternary complex concentration is very low (Theorell-Chance mechanism, Ref. 5), ferrocyanide would behave as a competitive inhibitor with ferricyanide as variable substrate. Thus, the product inhibition pattern as shown in Table I strongly favors the conclusion that the human NADH: (acceptor) oxidoreductase catalyzed reaction follows an ordered bi-bi mechanism. The observed product inhibition patterns are not compatible with rapid-equilibrium random bi-bi, ping-pong bi-bi or Theorell-Chance ordered bi-bi reaction mechanism.

Substrate inhibition by ferricyanide

When the initial rate equation, based on the ordered bi-bi mechanism of reaction was compared with the experimental data it was observed that the substrate inhibition effect of ferricyanide caused a pronounced deviation from

TABLE I

PRODUCT INHIBITION PATTERNS

Var, variable substrate. C, competitive inhibition. NC, non-competitive inhibition.

Inhinitor	(mM)	NADH	Ferricyanide	Inhibition pattern
NAD^+	2.0	Var	0.33	C
NAD^+	5.0	Var	0.33	C
NAD^+	1.0	0.5	Var	NC
Ferrocyanide	100.0	1.0	Var	NC
Ferrocyanide	100.0	Var	0.1	NC

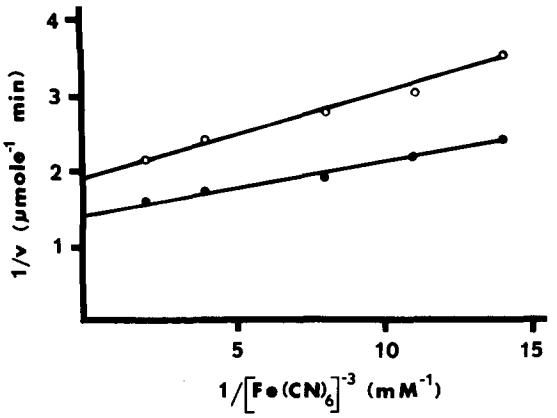

Fig. 2. Non-competitive product inhibition effect of ferrocyanide on NADH : ferricyanide oxidoreductase activity. The concentrations of ferrocyanide were: ○ (●———●), 100 mM (○———○). Concentration of NADH was 1.0 mM; enzyme concentration 2 μg/ml.

normal ordered bi-bi kinetic behavior (see below). Therefore, for deriving the kinetic rate equation of the enzymic reaction it is necessary to consider the substrate inhibition effect of ferricyanide as an essential element in the kinetic mechanism. In order to explore the substrate inhibitory effect of ferricyanide, a Lineweaver-Burk plot was examined with ferricyanide as the variable substrate. As shown in Fig. 3, the substrate inhibitory effect was dependent on NADH concentration. At high concentration of NADH, ferricyanide exhibited only a slight substrate inhibition effect. At low concentration of NADH, the inhibitory effect of ferricyanide was enhanced as evidenced by increasing deviation from linearity.

In order to further explore the substrate inhibitory effect of ferricyanide, a Lineweaver-Burk plot was examined with NADH as the variable substrate. In

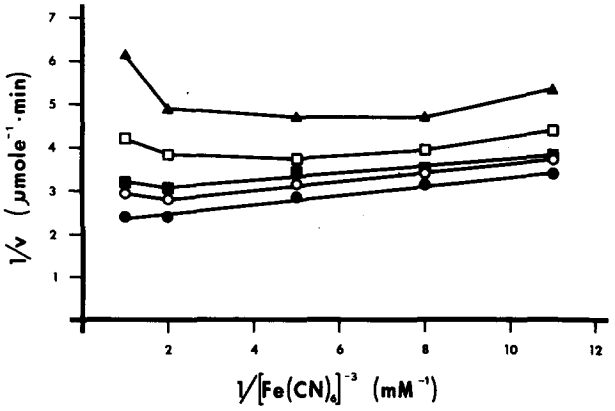

Fig. 3. Double-reciprocal plot with ferricyanide as the variable substrate. The concentrations of NADH were held constant at 1.0 mM (●———●), 0.25 mM (○———○), 0.125 mM (■———■), 0.067 mM (□———□) and 0.033 mM (▲———▲); enzyme concentration 1.3 μg/ml.

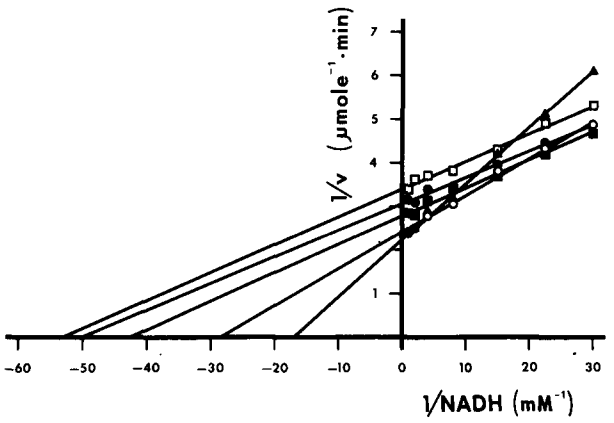

Fig. 4. Double-reciprocal plot with NADH as the variable substrate. The concentrations of ferricyanide were held constant at 1.0 mM (▲———▲), 0.5 mM (○———○), 0.2 mM (■———■), 0.125 mM (●———●), 0.091 mM (□———□); enzyme concentration 1.3 μg/ml.

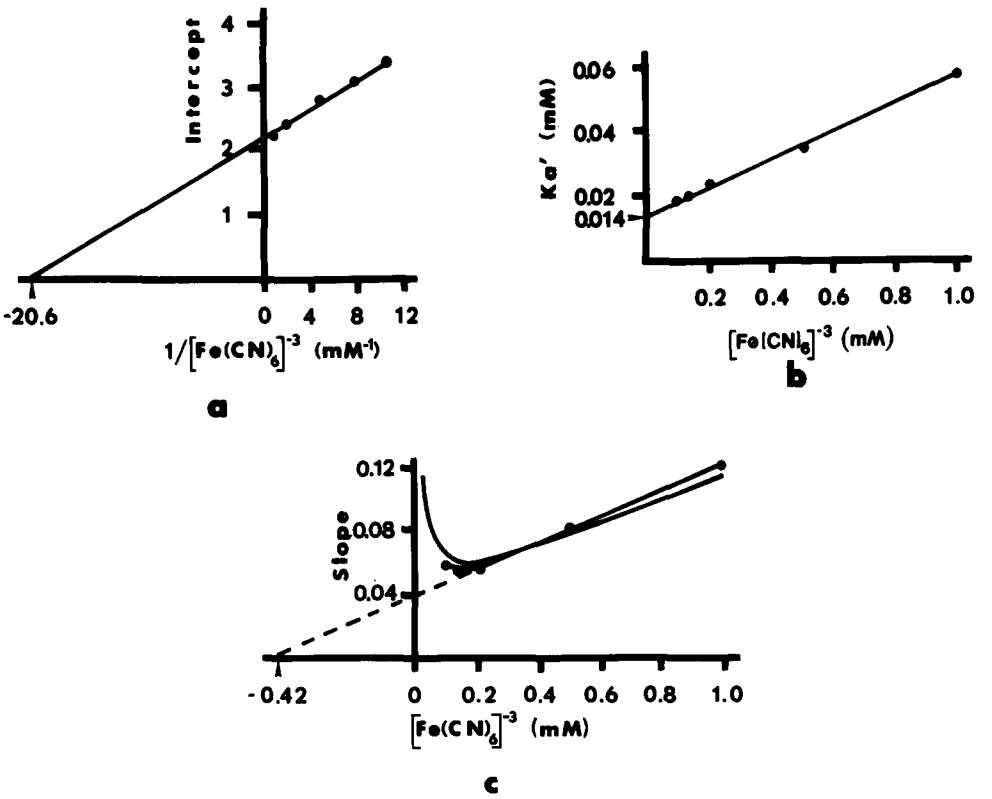

Fig. 5. Derivation of Michaelis-Menten and dissociation constants for ferricyanide and NADH using the data of Fig. 4. (a). The plot of the ordinate intercepts from Fig. 4 vs. the reciprocals of ferricyanide concentration. (b). The plot of K'_a (the negative reciprocals of the intercepts at the abscissa), obtained from Fig. 4 vs. ferricyanide concentrations. (c). The plot of the slopes from Fig. 4 vs. ferricyanide concentration. ———, curve obtained based on Eqn. 2; ●———●, curve obtained from Fig. 4; - - - - - -, extrapolation of the linear portion of the curve derived from Fig. 4.

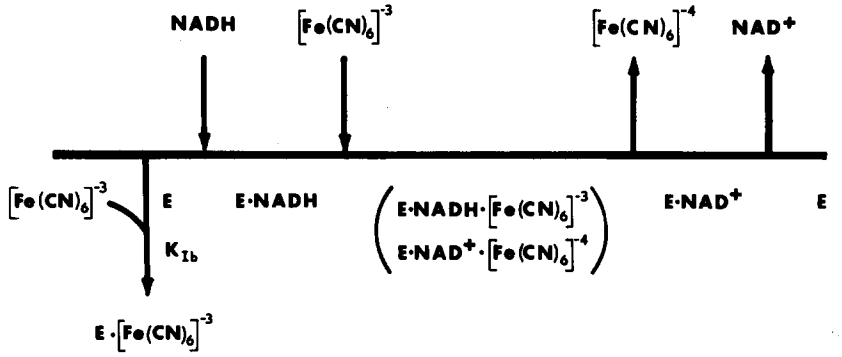

Fig. 6. The proposed reaction mechanism for NADH : ferricyanide oxidoreductase with the competitive substrate inhibition effect of ferricyanide.

cotrast to ferricyanide, NADH did not exhibit any substrate inhibition effect as shown in Fig. 4, where linear plots were obtained pertaining to five concentrations of ferricyanide. Due to the ferricyanide substrate inhibition effect, the linear functions did not show a common point of convergence as is expected for an ordered bi-bi mechanism. However, by examining the intercept of the ordinate (Fig. 4), it was observed that the apparent V increased as the ferricyanide concentration increased despite the substrate inhibitory effect of ferricyanide.

A plot of the apparent V (intercept of ordinate) and reciprocal of ferricyanide concentration was linear, as expected for normal bi-bi ordered reaction mechanism (Fig. 5). This is the most specific characteristic feature of competitive substrate inhibition [6,7] and can be utilized for differentiating competitive substrate inhibition from non-competitive or uncompetitive substrate inhibition effects. For non-competitive or uncompetitive substrate inhibition by ferricyanide, the expected curve for such a plot would be a hyperbola [4]. In other words, the non-competitive or uncompetitive substrate inhibition effect of ferricyanide would not be overcome by a saturating NADH concentration. Thus, the substrate inhibition effect of ferricyanide could be described by its competition with NADH for binding to the free enzyme in forming an inactive enzyme-ferricyanide binary complex.

The Michaelis-Menten constant for ferricyanide was obtained from the plot described above employing the intercept of the ordinate from Fig. 4 vs. the reciprocal of the concentration of ferricyanide (Fig. 5a). From the intercept of the abscissa of Fig. 5a, it was estimated that the Michaelis-Menten constant for ferricyanide is 0.049 mM.

The Michaelis-Menten constant for NADH (K_a) can be obtained from the secondary plot employing the apparent Michaelis-Menten constant K'_a (negative reciprocal of the intercept at the abscissa, Fig. 4) vs. ferricyanide concentration. The linearity of the plot (Fig. 5b) indicated that the Michaelis-Menten constant for NADH (K_a) can be related to ferricyanide concentration [B] by fitting into Eqn. 1 [6]:

$$K'_a = K_a \left(1 + \frac{[B]}{K_{Ib}}\right) \quad (1)$$

where K_{Ib} is the dissociation constant of the enzyme-ferricyanide binary complex. From the intercept of the ordinate and slope of Fig. 5b, it can be determined that K_a and K_{Ib} were 0.014 and 0.37 mM, respectively. In order to test further the validity of the various parameters obtained by this approach, the slopes obtained from Fig. 2 were compared with that directly calculated from Eqn. 2:

$$\frac{1}{V} = \frac{1}{V}\left\{1 + \frac{K_a}{[A]}\left(1 + \frac{[B]}{K_{Ib}}\right) + \frac{K_b}{[B]} + \frac{K_{ia}K_b}{[A][B]}\left(1 + \frac{[B]}{K_{Ib}}\right)\right\} \quad (2)$$

Where [A] is the concentration of NADH and K_{ia} is the dissociation constant of the enzyme-NADH binary complex. The term related to the free enzyme concentration is multiplied by a factor of $(1 + [B]/K_{Ib})$ accounting for the dead-end enzyme-ferricyanide complex. However, the estimation of the slopes from Fig. 4 using Eqn. 2 requires also the estimation of K_{ia}. The plot of slope against concentration was linear at high ferricyanide concentrations; the intercept of this line at the abscissa, X_{SB}, is given by:

$$-X_{SB} = K_{Ib} + \left(\frac{K_{ia}}{K_a}\right)K_b \quad (3)$$

Where $-X_{SB} = 0.42$ mM and it can be calculated that $K_{ia} = K_a = 0.014$ mM. Thus, it is possible to compare the experimentally determined slope with that obtained from Eqn. 2 as shown in Fig. 5c. The close fit of the model to the experimental data strongly supports the view that ferricyanide was indeed a competitive substrate inhibitor.

Reaction mechanism

The competitive substrate inhibition effect is a specific phenomenon for ordered multiple-substrate enzyme systems. Thus, the identification of ferricyanide as a competitive substrate inhibitor is consistant with the proposed ordered bi-bi mechanism. As derived from product inhibition patterns and substrate inhibition kinetics by ferricyanide, the mechanism of the enzyme reaction with ferricyanide as electron acceptor can be written in Cleland's notation [8] as shown in Fig. 6 where the competitive substrate inhibition effect of ferricyanide is also indicated.

Homeomorphism (different reaction mechanisms sharing the same form of rate equation) is one of the difficulties generally encountered in determining enzyme reaction mechanisms. For example, the ordered bi-bi mechanism and rapid-equilibrium random bi-bi mechanism have been shown to share the same form of initial rate equation [9]. The presence of a substrate inhibition effect of ferricyanide caused an alteration of the enzyme reaction from a normal kinetic pattern. The multiplication of the factor $(1 + [B]/K_{Ib})$ to the terms related to the free enzyme concentration resulted in an initial rate equation which is in an unsymmetrical algebraic form. Thus, the competitive substrate inhibition effect prevents the homeomorphism and excludes the possible rapid-equilibrium random bi-bi mechanism, since these two phenomena are mutually exclusive.

Although ferricyanide is a convenient substrate for assay of the enzyme activity, the physiological substrate for the enzyme is not known. Recently,

Orringer and Roer [10] have suggested that the enzyme may be a part of an ascorbate-mediated transmembrane-reducing system of the human erythrocyte. However, a preliminary study undertaken in this laboratory indicated that the enzyme could not utilize dehydroascorbate directly as an electron acceptor. Cytochrome b_5 has been shown to be an intermediate electron acceptor for human erythrocyte cytosol methemoglobin reductase [11,12] and NADH: (acceptor) oxidoreductase is able to use cytochrome *c* as an electron acceptor [13]. The enzyme described in this paper may have a role in the conversion of methemoglobin to hemoglobin, although in a preliminary study a direct conversion catalyzed by the enzyme could not be demonstrated. When the natural substrate for reduction by the enzyme is known it will be instructive to apply the kinetic procedures delineated in this paper to that substrate, to establish if it shows the same enzyme inhibition as does ferricyanide.

Acknowledgements

This study was supported by the resources of the Oklahoma Medical Research Foundation. I would like to thank Dr. P. Alaupovic for his interest and support of this work, Dr. P. Weech for his valuable criticism and suggestions in the preparation of this manuscript. The technical assistance of Mr. R. Hatcher and Ms. D. Downs is also appreciated. I would also like to thank Mrs. C.A. Beaver for typing this manuscript.

References

1 Zamudio, I., Cellino, M. and Canessa-Fischer, M. (1969) Arch. Biochem. Biophys. 129, 336—345
2 Wang, C.-S. and Alaupovic, P. (1978) J. Supramol. Struct. 9, 1—14
3 Wang, C.-S. and Smith, R.L. (1975) Anal. Biochem. 63, 414—417
4 Cleland, W.W. (1970) Enzymes 2, 1—65
5 Theorell, H., Chance, B. (1951) Acta Chem. Scand. 5, 1127—1144
6 Wang, C.-S. (1977) Eur. J. Biochem. 78, 569—574
7 Segel, I.H. (1974) in Enzyme Kinetics, pp. 819—824, John Wiley and Sons, New York
8 Cleland, W.W. (1963) Biochim. Biophys. Acta 67, 104—137
9 Dalziel, K. (1957) Acta Chem. Scand. 11, 1706—1723
10 Orringer, E.P. and Roer, M.E.S. (1979) J. Clin. Invest. 63, 53—58
11 Passon, P.G. and Hultquist, D.E. (1972) Biochim. Biophys. Acta 275, 62—73
12 Tomoda, A., Yubisui, T., Tsuji, A. and Yoneyama, Y. (1979) J. Biol. Chem. 254, 3119—3123
13 Zamudio, I. and Canessa, M. (1966) Biochim. Biophys. Acta 120, 165—169