KINETICS AND SPECIFICITY OF GLUCOSE DEHYDROGENASE FROM *BACTERIUM ANITRATUM*

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SUMMARY

Soluble glucose dehydrogenase from *Bacterium anitratum* acts on a number of aldose sugars, with decreasing rate as the structure is removed from that of D-glucose.

Linear double reciprocal plots were obtained for glucose with a series of acceptor (2,6-dichlorophenolindophenol) concentrations, and these were in the main parallel. The independence of the slope on the acceptor concentration suggests a mechanism involving a reducible group on the enzyme.

Deviation from parallel linearity at high concentrations of substrate and acceptor could be interpreted as mutual competitive inhibition between substrate and acceptor. Atebrin was shown to inhibit through competition with the acceptor.

MICHAELIS constants, maximal velocities, and inhibition constants for the reaction are presented and discussed in terms of the mechanism proposed.

METHODS AND MATERIALS

The enzyme preparations used were prepared as described in the preceding paper[1]. The initial reaction rates were determined in 0.05 *M* phosphate buffer of pH 6.0 in a thermostated Beckman DU at 25°. All velocity data given are per mg protein.

The carbohydrates and other substances tested were obtained as indicated earlier, and from the following firms: D-xylose, L-xylose, D-ribose, D-fructose and D-galactose from Hoffmann-La Roche & Co; L-arabinose from Merck AG; D-arabinose from Nutritional Biochemicals Corporation; D-glucose-6-phosphate from Sigma Chemical Company; DL-glyceraldehyde from California Foundation for Biochemical Research. D-2-desoxyglucose was a gift from Dr. O. WALAAS.

RESULTS AND DISCUSSION

Specificity

The relative reaction rates with 0.02 *M* solutions of some aldoses and $4.0 \cdot 10^{-5}$ *M* DIP are given in Table I. In addition to these compounds, D-fructose, D-glucose-6-

Abbreviation: DIP, 2,6-dichlorophenolindophenol.

phosphate, sorbitol, and mannitol were tested and found not to be attacked. Within the aldoses, the dehydrogenase exhibits a rather wide, but characteristic, specificity pattern. Based on the limited number of substances tested, it appears that in order to have a relative activity above 50%, the configuration must start with (+ —).

TABLE I

SPECIFICITY

Substrate	*Configuration*	*% of maximal activity*	
		Sol. enz.	*Part. enz.*
D-glucose	+ — + +	100	100
D-galactose	+ — — +	66	
L-arabinose	+ — —	51	100
D-mannose	— — + +	30	
D-xylose	+ — +	27	100
D-ribose	— — —	7.5	
D-2-desoxyglucose	o — + +	4.5	
D-arabinose	— + +	1.6	1.5
DL-glyceraldehyde	+ —, — +	1.0	
L-xylose	— + —	o	o

It is interesting to note that for the pentoses tested with the soluble enzyme, it is more important that this (+ —) is followed by a (—) as in L-arabinose, than by a (+), as in D-xylose, although the latter appears more akin to D-glucose in structure.The pattern for the soluble and the particulate enzyme is the same in that inversion of the first two hydroxyls to (— +) leads to virtual or complete disappearance of activity. The particle, however, fails to distinguish between D-glucose, L-arabinose, and D-xylose under the conditions used. One is therefore led to pursue the specificity relationships further in terms of MICHAELIS constants and maximal velocities.

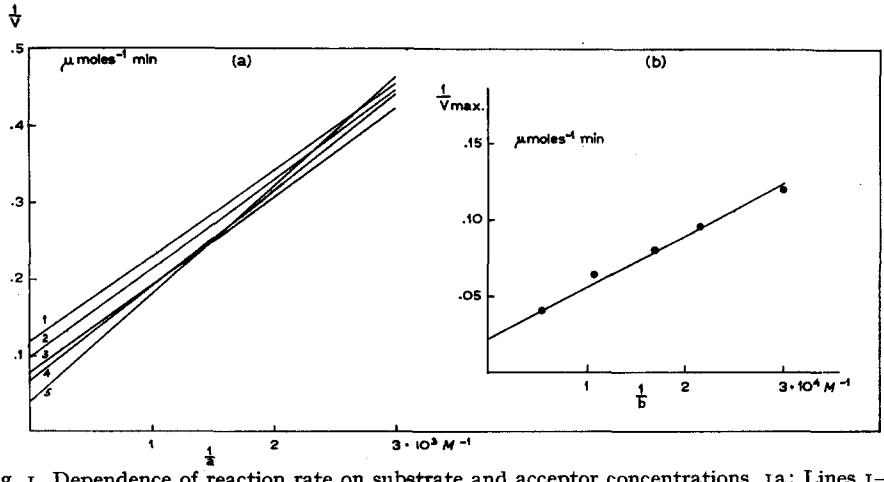

Fig. 1. Dependence of reaction rate on substrate and acceptor concentrations. 1a: Lines 1–5 are reproductions of double reciprocal plots determined by five glucose concentrations from 3.3 to $200 \cdot 10^{-4}$ *M*. The DIP concentrations were 3.3, 4.6, 5.8, 9.2 and $18.2 \cdot 10^{-5}$ *M* respectively. 1b: The intercepts on the $1/V$ axis of Fig. 1a are here plotted against $1/b$. The enzyme preparation used had a specific activity of 44 units/mg.

Kinetics of the glucose-DIP reaction

The initial rate of reaction was determined for a series of DIP concentrations, each with a series of glucose concentrations. In double reciprocal plots a system of straight lines appeared. The lines are reproduced in Fig. 1a. Fig. 1b is a plot of the reciprocal maximal velocities thus obtained against the inverse DIP concentrations. The linear relationships obtained show that the data conform to the two-substrate rate equation of DALZIEL[2]

$$\frac{(E)_0}{V} = \phi_0 + \frac{\phi_1}{a} + \frac{\phi_2}{b} + \frac{\phi_{12}}{ab} \qquad (1)$$

where a and b in the present case are the initial concentrations of glucose and DIP respectively, and $(E)_0$ is the concentration of active enzyme sites.

ϕ_{12} is zero for DIP concentrations in the range 1.0 to $6.0 \cdot 10^{-5}$ M, as shown by the fact that the $1/V$ *versus* $1/a$ lines are parallel (Fig. 1a, 2a). The parameter may well be zero in the whole range measured, which extends to $18 \cdot 10^{-5}$ M. The slope in this higher region increases, rather than decreases, as would be expected if ϕ_{12} was not zero. This change in slope is due to an inhibition by DIP. In addition to this inhibition, revealing itself particularly at high DIP and low glucose concentrations, one also observes (Fig. 2) an inhibition for high glucose and low DIP concentrations. If these

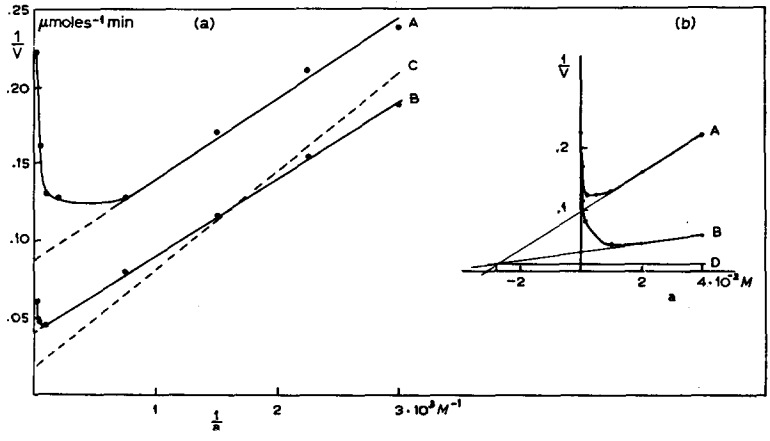

Fig. 2. Inhibition by excess substrate and acceptor. 2a: Curve A and B correspond to DIP concentrations of 1.0 and $4.6 \cdot 10^{-5}$ M respectively. The enzyme preparation had a specific activity of 108 units/mg. Line C has been transposed from Fig. 1a, where it had number 5. 2b: $1/V$ is here for the same experiment replotted against the substrate concentration. Line D represents the ideal velocity for excess substrate and acceptor, 112 μmoles $\times$ min^{-1} $\times$ mg^{-1}.

inhibitions are competitive, as is indicated by the nature of the curves, they may be taken into account in the equation by multiplying the ϕ_1 term with $(1 + b/K_{ib})$, and the ϕ_2 term with $(1 + a/K_{ia})$, K_{ia} and K_{ib} being inhibition constants (Eqn. (2)). These factors are essentially unity for a large enough portion of the data of Fig. 1 to allow $1/(E)_0 \times \phi_0$, ϕ_1, and ϕ_2 to be computed.

From the slope of the parallel lines of Fig. 1a, we find $\phi_1/(E)_0 = 1.16 \cdot 10^{-4}$ μmoles^{-1} $\times$ min $\times$ M. From the slope of Fig. 1b: $\phi_2/(E)_0 = 3.4 \cdot 10^{-6}$ μmoles^{-1} $\times$

min $\times$ M, and from the intercept of the same figure: $\phi_0/(E)_0 = 2.2 \cdot 10^{-2}$ μmoles^{-1} $\times$ min. The MICHAELIS constant for glucose, DIP in excess, was $\phi_1/\phi_0 = 5.3 \cdot 10^{-3}$ M, the corresponding constant for DIP $\phi_2/\phi_0 = 1.6 \cdot 10^{-4}$ M, and the maximal velocity per mg of this particular dehydrogenase preparation $(E)_0/\phi_0 = 45$ μmoles $\times$ min^{-1}, as compared to 400 μmoles $\times$ min^{-1} $\times$ mg^{-1} for the most active preparation isolated so far. These MICHAELIS constants and maximal velocities are ideal ones, as the inhibitions have not been taken into account.

Specificity analysis

Apparent MICHAELIS constants ($K_M^{ap} = \phi_1/(\phi_0 + \phi_2/b)$) and maximal velocities ($V_{max}^{ap} = (E)_0/(\phi_0 + \phi_2/b)$) obtained with L-arabinose, D-xylose, and D-glucose for the soluble and the particulate enzyme are compared in Table II. Whereas the various MICHAELIS constants for the soluble enzyme have been found identical, within

TABLE II

SPECIFICITY CONSTANTS

A soluble enzyme preparation with specific activity of 31 units/mg was used, and the particulate form had 0.6 units/mg. DIP was present in a concentration of $4.0 \cdot 10^{-5}$ M. V_{max}-values are given as μmoles $\times$ mg^{-1} $\times$ min^{-1}.

Substrate	*Soluble enzyme*		*Particulate enzyme*	
	K_M^{ap}	V_{max}^{ap}	K_M^{ap}	V_{max}^{ap}
	($M \times 10^3$)		($M \times 10^4$)	
D-glucose	1.1	7.0	2.3	0.08
L-arabinose	22	7.0	10.0	0.08
D-xylose	51	7.0	3.0	0.08

experimental error, for differing dehydrogenase preparations, there was a certain variation in the constants for the particle. On an average for three preparations, the glucose, arabinose, and xylose constants were 1.5, 7.6, and 2.9 $\times$ 10^{-4} M respectively. Individual data deviated from these figures with up to 50%.

It is apparent that the higher rate of oxidation of L-arabinose as compared to D-xylose with the soluble enzyme is due to a lower ϕ_1-value, suggesting a higher affinity of the enzyme for this substrate, and not to a difference in reaction rate once saturation has been assured. Identical V_{max}^{ap}-values were also obtained with the particulate enzyme for all three substrates. The K_M^{ap}-values have, however, been strikingly reduced, and not to the same degree. D-xylose here appears as a better substrate than L-arabinose, and as only little inferior to D-glucose. An approximate determination of ϕ_2/ϕ_0 showed that this ratio in the particulate form was five times lower than for the soluble enzyme. There has thus been a relatively larger increase in ϕ_0 than in ϕ_2. ϕ_1 has in addition been affected in a specific manner not eliminated by division with ϕ_0, as shown by the changes in K_M^{ap} for the substrates.

Table II serves to emphasize the dependence of specificity data on the substrate concentration used. Had the data of Table I been collected at a lower substrate level, the relative preference for glucose would have appeared greater.

Inhibitions

From the data given in Fig. 2 approximate values for the inhibition constants K_{ia} and K_{ib} referred to above may be calculated. The positions of the $1/V$ *versus* $1/a$ lines for the highest DIP concentrations in relation to the parallel lines obtained at lower concentrations are such (Fig. 1a, 2a) as would be expected for a competitive inhibition. The ratio of the slopes for lines C and B in Fig. 2a is 1.25. Equating this with $(1 + b/K_{ib})$, which is valid both for competitive and noncompetitive inhibition, a value of $7 \cdot 10^{-4}$ M for K_{ib} is obtained. The inhibition constant is thus 7-fold lower than the glucose MICHAELIS constant given above.

In order to obtain K_{ia}, the points on curve A and B in Fig. 2a were, following DIXONS method[3], replotted as $1/V$ *versus* a (Fig. 2b). The extrapolated straight line sections cross each other at a $1/V$ value in good agreement with the expected $1/V_{max}$, and at a negative glucose concentration of $2.8 \cdot 10^{-2}$ M, directly giving K_{ia}.

Because of these inhibitions, the maximal velocity $(E)_0/\phi_0$ can not be attained. Analysis of Eqn. (2)

$$\frac{(E)_0}{V} = \phi_0 + \frac{\phi_1}{a}\left[1 + \frac{b}{K_{ib}}\right] + \frac{\phi_2}{b}\left[1 + \frac{a}{K_{ia}}\right] \qquad (2)$$

reveals that $(E)_0/V\phi_0$ asymptotically approaches $V_{max}/V'_{max} = 1 + 2\sqrt{\frac{\phi_1\phi_2}{\phi_0^2 K_{ia} K_{ib}}}$ as its minimal value when a and b are increased in the optimal ratio $a/b = \sqrt{\frac{\phi_1 K_{ia}}{\phi_2 K_{ib}}}$. Inserting the values found above, V_{max}/V'_{max} becomes 1.42, and a/b 36.5. 70 % of the ideal maximal velocity may thus be reached, competitive inhibition being assumed.

It was of special interest to study the nature of the atebrin inhibition reported in the preceding paper. Fig. 3 gives the result of a test of three atebrin concentrations with three levels of DIP. The distribution of the points indicates a competition between atebrin and DIP. The intercept on the $1/V$ axis gives the expected V_{max}. From the ratio of the slopes of the two top lines to the lower one, K_i-values of 3.8 and $4.0 \cdot 10^{-3}$ M respectively are obtained.

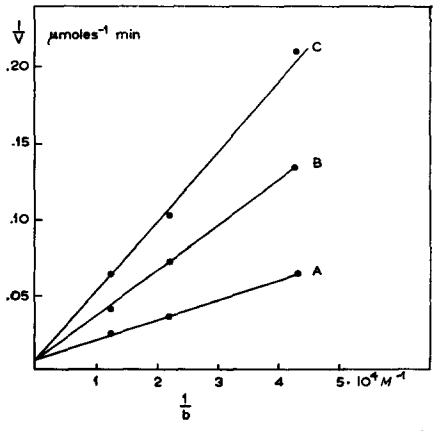

Fig. 3. Competitive inhibition by atebrin. The concentrations of atebrin were for the three series A, B, and C: 0, 4.4, and $8.8 \cdot 10^{-3}$ M respectively. The enzyme preparation was the same as in Fig. 2.

DISCUSSION OF MECHANISM

It is hasardous to suggest a mechanism for the reaction on the basis of the data presented. If one makes the assumption, however, that ϕ_{12} really is zero, and not merely too small to be detected, one is directed to the type of mechanism where the enzyme is first reduced by the substrate and subsequently reoxidized by the acceptor. Among the enzyme reactions of the general form $A + B \rightleftharpoons C + D$ discussed by ALBERTY[4,5], this type is the only one which fulfils the criteria of $S_{12} = 0$; $\phi_0, \phi_1, \phi_2 \neq 0$. It may be formulated as follows:

$$SH_2 + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} SH_2E \overset{k_3}{\rightleftharpoons} S + EH_2 \tag{3}$$

$$EH_2 + A \underset{k'_2}{\overset{k'_1}{\rightleftharpoons}} EH_2A \overset{k'_3}{\rightleftharpoons} E + AH_2 \tag{4}$$

Initial steady state treatment of this set of reactions leads, as shown earlier[4,6], to the following expression:

$$\frac{(E)_0}{V} = \frac{1}{k_3} + \frac{1}{k'_3} + \frac{k_2 + k_3}{k_1 k_3 a} + \frac{k'_2 + k'_3}{k'_1 k'_3 b} \tag{5}$$

This is of the same form as the empirical rate expression (1) above, with $\phi_{12} = 0$ as the characteristic feature.

The change noted in the ϕ_2/ϕ_0 ratio in going from the soluble to the particulate enzyme could be explained by a decrease in k'_3 alone, permitting the constants of the first step (3) to be the same in the two forms of the enzyme. The variable reductions in ϕ_1 observed show, however, that also the latter constants are affected. If the "unitary" hypothesis[1] is to be retained, it must be admitted that the separation of the primary dehydrogenase from the particle, or the incorporation of it into the particle, has resulted in some modifications of the place of substrate attachment, as well as of the reoxidation mechanism.

If one assumes no connection to exist between the sets of data for the soluble and the particulate enzyme, it is possible to write a simpler mechanism that still conforms to the observed rate law. This is done by omitting the MICHAELIS complex in the second step (4). Denoting the forward rate constant for the new second step k_4, the new rate expression will be

$$\frac{(E)_0}{V} = \frac{1}{k_3} + \frac{k_2 + k_3}{k_1 k_3 a} + \frac{1}{k_4 b} \tag{6}$$

k_3, k_4, and $K_M = (k_2 + k_3)/k_1$ could then be obtained directly from the ϕ-parameters, once the purity of the enzyme, and thus $(E)_0$, was known.

Further evidence as to the nature of the mechanism will have to be sought through chemical studies of the enzyme, with possible identification of a group that can be responsible for the postulated reduction–oxidation.

NOTE ADDED IN PROOF

The reducing disaccharides maltose, lactose, and cellobiose were attacked almost as readily by the soluble enzyme as D-glucose, their K_m values being 4.7, 3.3, and $2.2 \cdot 10^{-3}$ M. With melibiose, a K_m of approximately 0.2 M was found.

The data reported in the paper refer to mutarotational equilibrium mixtures. For glucose it could be shown that the β-anomer is preferentially attacked: when the reaction was started by the addition of 10 μl of a freshly prepared cold 0.1 *M* solution of glucose, the reaction rate in the first half minute was only 10 % of that observed after the reaction had proceeded at 25° for 6 min.

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