INTERACTION BETWEEN GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE AND DPNH-CYTOCHROME REDUCTASE*

by

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Glyceraldehyde phosphate dehydrogenase (GPD***) isolated from rabbit muscle and repeatedly recrystallized contains 2 moles of bound DPN per mole (120,000 g) of enzyme protein[1,2,3]. This bound nucleotide cannot be removed by dilution or dialysis, but equilibrates freely with added, radioactive DPN[3]. Recent studies in the laboratories of VELICK[3,4,5], RACKER[6,7] and BOYER[8] on the mode of action of this enzyme, which constitutes the first, and so far the only example of a soluble, pure conjugated pyridinoprotein, have indicated that the mechanism of enzymic catalysis in this instance may be represented by the following series of equations:

1. $2\ RCHO + (HS)_2\text{–GPD–}(DPN^+)_2 \rightleftharpoons (RCHOHS)_2\text{–GPD–}(DPN^+)_2$§
(RCHO = glyceraldehyde or glyceraldehyde-3-phosphate)
2. $(RCHOHS)_2\text{–GPD–}(DPN^+)_2 \rightleftharpoons (RCOS)_2\text{–GPD–}(DPNH)_2 + 2\ H^+$
3. $(RCOS)_2\text{–GPD–}(DPNH)_2 + 2\ DPN^+ \rightleftharpoons (RCOS)_2\text{–GPD–}(DPN^+)_2 + 2\ DPNH$
4. $(RCOS)_2\text{–GPD–}(DPN^+)_2 + HR' \rightleftharpoons RCOR' + (HS)_2\text{–GPD–}(DPN^+)_2$
(R'H = phosphate or arsenate)

For the purposes of this communication equations 3 and 4 are of primary interest. In the course of reaction 4 what may be called the substrate acceptor sites of the enzyme

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*** The following abbreviations will be used: GPD (glyceraldehyde phosphate dehydrogenase), DPN^+ or DPN (oxidized diphosphopyridine nucleotide) DPNH (reduced DPN), E_λ (extinction = log I_0/I, as determined in a Beckman model DU spectrophotometer, at a wavelength λ), tris (tris(hydroxymethyl)aminomethane), diol (2-amino-1-methyl-1,3-propanediol), EDTA (ethylenediaminetetraacetic acid), K_m' = Michaelis-Menten constant for one component in an enzymic reaction; the K_m' for the reaction A + Enzyme $\underset{k_2}{\overset{k_1}{\rightleftharpoons}}$ Enzyme — A $\xrightarrow{k_3}$ products is defined by the relation $K_m' = (k_2 + k_3)/k_1$, the dissociation constant K_d for the same component A is defined as k_2/k_1.

§ RACKER AND KRIMSKY[6] have postulated a different mechanism for steps 1 and 2, involving an aldehydolysis of a GPD–S–DPNH bond. Since this mechanism leads to the identical intermediate for step 3 no choice between the two need be made for the purposes of this discussion.

References p. 107.

are regenerated, while reaction 3 represents the regeneration of enzyme-bound, oxidized DPN. This process is ordinarily accomplished by interaction with external, added DPN^+, and when this is accompanied by the freeing of the acceptor sites (equation 4), gives rise to a completely regenerated active enzyme molecule, ready to undergo the catalytic cycle (reaction 1–4) once again. It is apparent from the equation, however, that a priori any other method which leads to a reoxidation of the bound DPNH might also be effective, and that if other paths for the regeneration of enzyme-bound DPN^+ exist, they should substitute for equation 3 in the manner of equation 5.

5. $(RCOS)_2\text{-GPD-}(DPNH)_2 \overset{\text{enzyme}}{\rightleftharpoons} (RCOS)_2\text{-GPD-}(DPN^+)_2 + 2\ H^+ + 4\ e^-$

Such a sequence of reactions might lead to an interaction of (phospho)glyceraldehyde and GPD with other enzymes and their substrates even in the *complete absence* of any externally added DPN^+, using only the two DPN^+ molecules bound to the GPD molecule.

The feasability of this approach had already been shown by CORI, VELICK AND CORI who demonstrated the interaction of phosphoglyceraldehyde, GPD, pyruvate and lactic dehydrogenase[2]. During the oxidative phase most cells maintain their DPN in the oxidized form by means of interaction with the cytochrome system. DPNH-cytochrome reductase, the enzyme responsible for the reoxidation of DPNH by cytochrome *c* has been isolated from animal tissues[9] and extensively purified[10]. It appeared of interest to determine whether direct interaction between GPD and DPNH-cytochrome reductase is possible.

The present report deals with several different ways of measuring this interaction:

a. In the presence of an excess of glyceraldehyde (or glyceraldehyde phosphate), but in the absence of added phosphate or arsenate, the overall reaction is limited by the amount of GPD present and may be represented by equations 6a or b depending on whether cytochrome or 2,6-dichlorophenol-indophenol is used as the terminal electron acceptor.

6a. $(RCOS)_2\text{-GPD-}(DPNH)_2 + 4$ cytochrome $(Fe^{+3}) \xrightarrow[\text{reductase}]{\text{cytochrome}} (DPN^+)_2\text{-GPD-}(SOCR)_2 + 4$ cytochrome $(Fe^{+2}) + 2\ H^+$

b. $(RCOS)_2\text{-GPD-}(DPNH)_2 + 2$ indophenol $+ 2\ H^+ \xrightarrow[\text{reductase}]{\text{cytochrome}} (DPN^+)_2\text{-GPD-}(SOCR)_2 + 2$ indophenol H_2

This may be termed the "stoichiometric" reaction, since no mechanism for the regeneration of the acceptor site is available.

b. In the presence of both substrate and arsenate, the over-all reaction is given by either equation 7a or 7b with the enzyme functioning catalytically.

7a. 3-phosphoglyceraldehyde $+ 2$ cytochrome $(Fe^{+3}) \xrightarrow[\text{arsenate}]{\text{GPD, cytochrome reductase}}$ 3-phosphoglycerate $+ 2$ cytochrome $Fe^{+2} + 2\ H^+$

b. 3-phosphoglyceraldehyde $+$ indophenol $\xrightarrow[\text{arsenate}]{\text{GPD, cytochrome reductase}}$ 3-phosphoglycerate $+$ indophenol H_2

This will be called the "catalytic" reaction.

References p. 107.

EXPERIMENTAL

Materials. PGD, 3 × recrystallized, was prepared by Dr. BOYER[8], according to the method of CORI *et al.*[1]. Several times recrystallized aldolase, free of isomerase was prepared by Dr. K. H. LING according to TAYLOR *et al.*[11], and fructose-1,6-diphosphate was a recrystallized commercial preparation, purified by Dr. W. BYRNE. These two preparations were made available to us through the generosity of Prof. HENRY A. LARDY of the Institute for Enzyme Research. DPN cytochrome reductase of specific activity 150 or better was prepared by the method of MAHLER *et al.*[10]. Diaphorase was purified according to STRAUB[12]. The DPN^+ and DPNH used were commercial preparations (Sigma Chemical Co., St. Louis, Mo.) of better than 90% purity by enzymic assay. The EDTA (Bersworth Chemical Co., Framingham, Mass.), Tris (Sigma Chemical Co.) and diol (recrystallized, Commercial Solvents Corp., Terre Haute, Ind.) and 2,6-dichlorophenolindophenol (Eastman), were commercial preparations.

Methods

Cytochrome c reduction (reductase activity). 1.0 mg cytochrome *c* (80% or better in the oxidized form), 33 μmoles of diol buffer pH 8.5, and the system responsible for generating the DPNH are placed in a total volume of 1.0 ml in 1 ml quartz cuvettes with a light path of 1.00 cm. The $E_{550.5}$ is read against a blank containing cytochrome *c*, water and buffer only. When no further changes are observed the reaction is started by the addition of the appropriate amount of cytochrome reductase and the increase in $E_{550.5}$ determined. If very accurate measurements are desired the experimental cuvette is read against a blank cell containing all the components of the former except cytochrome reductase.

Indophenol reduction (diaphorase activity). 10 μg of 2,6-dichlorophenolindophenol, 20 μmoles of Tris, pH 7.8 and the DPNH generating system are diluted to 1.0 ml. Dye reduction is followed spectrophotometrically at 600 mμ against a water blank. The reaction is started by the addition of reductase or diaphorase and the decrease in E_{600} is measured. The values so obtained are subtracted from those obtained with a blank containing no enzyme.

Protein concentration. The protein concentration of GPD was determined by using the relation E_{278} = mg/ml[8]. The concentration of the other proteins used was measured by the biuret reaction.

Extinction coefficients. The amount of cytochrome *c* reduced in micromoles, under the experimental conditions used equals $\Delta E_{550} \cdot 5.35 \cdot 10^{-2}$ [13], the amount of indophenol reduced equals $-\Delta E_{600} \cdot 6.25 \cdot 10^{-2}$, and the amount of DPNH produced or consumed is $\Delta E_{340} \cdot 16.1 \cdot 10^{-2}$ [14].

REACTION WITH GLYCERALDEHYDE

To test the hypothesis that GPD-$(DPNH)_2$ is capable of interaction with cytochrome *c* in the presence of reductase, glyceraldehyde was first used as a substrate, and the data of Fig. 1 were obtained. In the absence of arsenate (curve A), rapid reduction of cytochrome *c* is observed initially. After eight minutes this reaction is completed, and an equilibrium value has been approached; this constitutes the "stoichiometric" reaction. When arsenate is added at point 2, rapid reduction is resumed, with a rate identical to that of the initial rate in the absence of arsenate, indicating that the same step has again become rate limiting. If arsenate is present initially (curve B), the initial rate, identical with the linear rates observed previously is maintained over a considerable period of time,—the "catalytic" reaction.

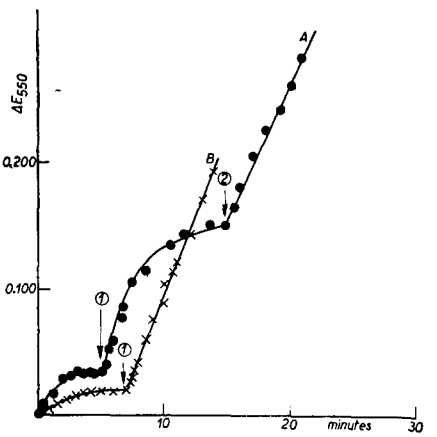

Fig. 1. Reaction with cytochrome *c*, glyceraldehyde as substrate.—The experimental cuvette for curve A contained in 1.0 ml at 22°: 30 μmoles of diol pH 8.5, 100 μmoles of D,L-glyceraldehyde, 1 mg of oxidized cytochrome *c*, 0.2 μmoles of EDTA, and 2.12 mμ moles of GPD, added at zero time; at point 1, 10 γ of reductase were added, and at point 2, 2 μmoles of arsenate. Curve B: similar to A, except that arsenate was added at zero time.

References p. 107.

REACTIONS WITH D-GLYCERALDEHYDE-3-PHOSPHATE

The use of glyceraldehyde as a substrate is attended by several drawbacks. Blanks are relatively high, the affinity of the substrate for GPD is low and so are the enzymic rates. The true substrate for the enzyme D-glyceraldehyde-3-phosphate was generated by the prior addition to the cuvette of fructose-1.6-diphosphate and aldolase, both in excess, to insure the presence of a continuous supply of the aldehyde. Under these conditions the "stoichiometric" reaction is exemplified by curves A and B of Fig. 2 for two different levels of GPD varying by a factor of 2. Once again the "stoichiometric" can be changed to the "catalytic" reaction by the addition of arsenate after equilibrium has been obtained (curve B). If arsenate is present initially (curve C), at a GPD level identical to that of curve A, the initial rate is maintained over a long period of time.

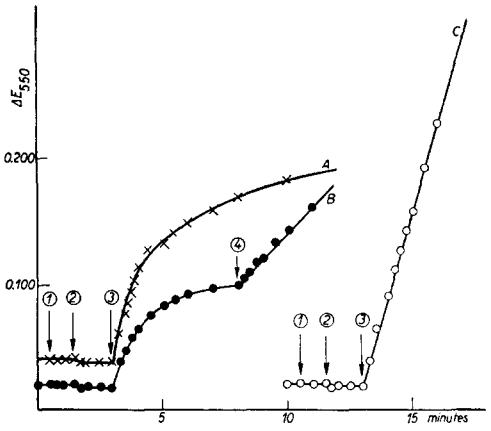

Fig. 2. Reaction with cytochrome *c*, D-glyceraldehyde-3-phosphate as substrate. —The experimental cuvette for curve A contained in 1.0 ml at 22°: 30 μmoles of diol pH 8.5, 1 mg of oxidized cytochrome *c* and 5 μmoles of fructose 1,6-diphosphate, at point 1, 5 γ of aldolase, at point 2, 4.80 mμ moles of GPD and at point 3, 10 γ of reductase were added. Experiment B was performed in a similar manner except that 2.40 mμ moles of GPD were used, at point 4, 2 μmoles of arsenate were added. Experiment C was similar to A, but 2 μmoles of arsenate were included at zero time.

REACTIONS WITH INDOPHENOL

The interaction between GPD-bound DPNH and electron acceptor, catalyzed by cytochrome reductase, is not confined to cytochrome *c* as oxidant. Dyes, such as 2.6-dichlorophenolindophenol can function equally well. Figure 3 shows the dependence of the "catalytic" indophenol reduction on the GPD level, while Fig. 4 summarizes corresponding data for the "stoichiometric" reaction. Once again, the addition of arsenate permits resumption of the catalytic cycle and changes the "stoichiometric" to the "catalytic" reaction.

Other respiratory enzymes capable of reoxidizing DPNH can take the place of cytochrome reductase. STRAUB's diaphorase[12] can

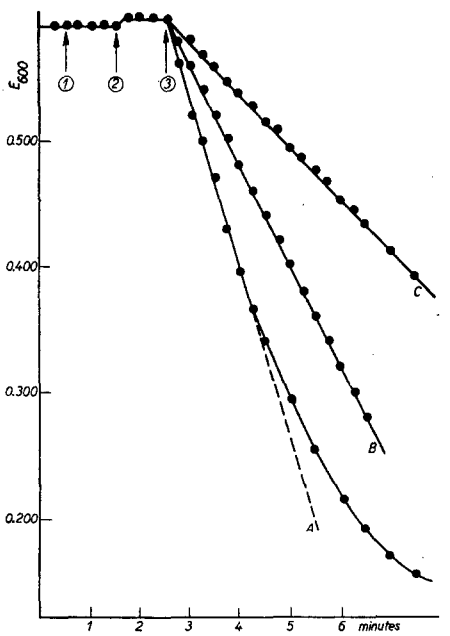

Fig. 3. Catalytic reaction with indophenol. Experiments similar to those of Fig. 2, except that 10 γ of indophenol was used as acceptor and 2 μmoles of arsenate was present in all experiments. The concentration of GPD was varied: cuvette A contained 4.80 mμ moles of GPD, cuvette B 2.40 mμ moles GPD, and curve C 0.88 mμ moles GPD.

References p. 107.

function with 2.6-dichlorophenolindophenol in a manner entirely analogous to that just described for reductase.

COMPARISON OF FREE AND BOUND DPNH

In absence of additional information the experiments just described are capable of more than one interpretation, as has already been suggested by CORI, VELICK AND CORI[2] in the case of the GPD-lactic dehydrogenase reaction. The most obvious explanation is that the small amount of dissociation of DPNH from the GPD-DPNH complex is sufficient for the reductions observed. This explanation is untenable on the basis of existing data: VELICK *et al.*[3] have estimated that the dissociation constant for the reaction GPD-DPN$^+$ $\rightleftharpoons$ GPD + DPN$^+$ is of the order of $2 \cdot 10^{-7}$ *M*. The dissociation constant for the complex GPD-DPNH may be assumed to be of the same order of magnitude; but

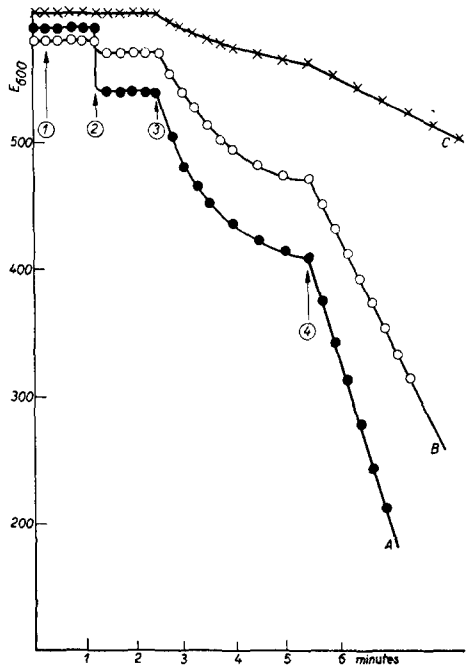

Fig. 4. Stoichiometric reaction with indophenol.—Experiments similar to those of Fig. 3, but with arsenate omitted at zero time, and added at point 4. Concentration of GPD for curve C was 1.20 mμmoles per ml, other concentrations as in Fig. 3.

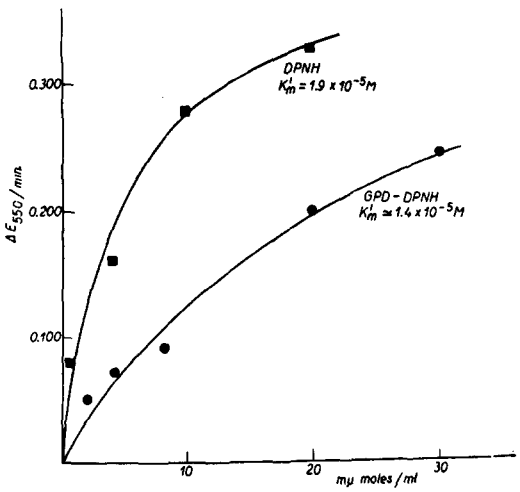

Fig. 5. Dependence of initial catalytic rate on DPNH concentration. — For the points with GPD the experimental conditions were those of Fig. 2, curve C with the GPD concentration varied as shown; the rate from 15 to 75 seconds was used as "V_0". For the points with free DPNH the conditions were similar but the GPD was omitted and DPN in the appropriate amounts was substituted; at zero time 1 γ of recrystallized alcohol dehydrogenase and 0.02 ml absolute ethanol was added.

References p. 107.

can be anywhere from 10^{-5} *M* or smaller without affecting the argument. The dissociation constant for the complex reductase-DPNH is not known, but the Michaelis constant for DPNH in the reductase reaction (concentration of DPNH necessary for half-maximal velocity) has been determined to be $2 \cdot 10^{-5}$ M. (The dissociation constant must be smaller than the Michaelis constant by definition***). Now the initial concentration of GPD-DPNH was of the order of 10^{-5} *M* in most of the experiments described. Thus it can be readily appreciated that dissociation of the DPNH-GPD complex with a dissociation constant (K_d) of 10^{-5} *M* or less,

$$9. \quad K_d = \frac{[\text{GPD}]\,[\text{DPNH}]}{[\text{DPNH-GPD}]} = \frac{X^2}{10^{-5} - X} \leq 10^{-5}$$

cannot possibly give rise to enough free DPNH (X), to account for the rates observed.

A more direct experimental demonstration is also possible. Fig. 5 compares the rates of the "catalytic" reaction at various GPD-DPNH levels with the corresponding rates for similar levels of free DPNH in the presence of all the components of the GPD-reductase system (*i.e.* buffer, arsenate, EDTA, hexose diphosphate and aldolase) in order to eliminate any effects due to these substances. The DPNH is generated catalytically by the presence of alcohol, alcohol dehydrogenase and DPN. Under these conditions the observed rates are of comparable orders of magnitude. Furthermore not only is the Michaelis constant for free DPNH determined under these somewhat unusual conditions identical with that determined previously under standard assay conditions[10] (*i.e.* $= 1.9 \cdot 10^{-5} M$), but the GPD-DPNH also gives rise to the typical curve, with a K'_m of approximately $1.4 \cdot 10^{-5} M$.

STOICHIOMETRY OF REACTION

Since the amount of reaction occurring in the "stoichiometric" reaction appears to be proportional to the total GPD present, both in the cytochrome *c* (Fig. 2) and indophenol reaction (Fig. 4), it is possible to establish the stoichiometry of reaction 5. The results of experiments of this sort are compiled in Table I.

TABLE I

STOICHIOMETRY OF GPD—ACCEPTOR REACTION

Experiment	*"Substrate"*	*Acceptor*	*mμ moles*		*R*	
			$(DPNH)_2$-GPD added	*Acceptor reduced*	*found*	*theory*
1	glyceraldehyde	cytochrome *c*	2.12	5.0	2.3	4.0
2	D-glyceraldehyde-3-phosphate	cytochrome *c*	2.12	4.20	2.0	4.0
3	D-glyceraldehyde-3-phosphate	cytochrome *c*	2.40	5.10	2.1	4.0
4	D-glyceraldehyde-3-phosphate	cytochrome *c*	4.80	8.55	1.8	4.0
5	D-glyceraldehyde-3-phosphate	indophenol	2.40	5.10	2.1	2.0
6	D-glyceraldehyde-3-phosphate	indophenol	0.88	1.87	2.1	2.0
7	D-glyceraldehyde-3-phosphate	indophenol	4.40	8.03	1.8	2.0
8	D-glyceraldehyde-3-phosphate	first cytochrome *c*, followed by indophenol	4.80	8.5 (cytc) 7.0 (indoph)		

Conditions: experiment 1—Fig. 1, curve A; experiments 2, 3—Fig. 2, curve B; experiment 4—Fig. 2, curve A; experiments 5, 6, 7—Fig. 4, curves B, C and A respectively. For experiment 8, two parallel cuvettes containing the components of the experiments of Fig. 2—curve A were set up. When equilibrium had been reached, the usual amount of indophenol was added to one of the cuvettes and the reduction of the dye measured, using the other cuvette as a blank.

It is apparent that the ratio (R) moles acceptor reduced ÷ moles enzyme-$(DPNH)_2$ present equals two in experiments 1 to 7. Inspection of equations 5a and b shows that this ratio is the correct one for indophenol and GPD-$(DPNH)_2$. If is only *half* that expected for cytochrome *c*, however. This is an unexpected result. It is conceivable that under the conditions of the experiment only one of the two bound DPNH molecules becomes actually reduced by its substrate. This explanation cannot account for the difference between two *oxidizing* agents as observed here. Another alternative would be to postulate a difference in steric accessibility between the two sites holding bound DPNH on GPD. Since *not* reduction of DPN-cytochrome reductase, but its reoxidation

References p. 107.

by the eventual acceptor is the point at issue here, one of the implications of this assumption is that there is close steric interaction not only between GPD-$(DPNH)_2$ and DPN-cytochrome reductase, but between these two enzymes and cytochrome *c* as well. In order to test this hypothesis, experiment 8 was performed: 4.80 mμ moles GPD-$(DPNH)_2$, corresponding to 19.20 reducing mμ equivalents were allowed to interact with cytochrome *c* in the usual manner. 8.5 mμ moles of reduced cytochrome *c* were formed at equilibrium, corresponding to 8.5 reducing mμ equivalents, leaving 10.7 reducing mμ equivalents unused. Indophenol was then added and an additional 7 mμ moles of dye were reduced corresponding to 14 mμ equivalents reducing power. Thus it appears as if approximately half of the reducing equivalents, or one DPNH per GPD molecules is accessible and thus oxidizable by cytochrome, in the presence of reductase, while both DPNH molecules are oxidizable by indophenol, a smaller molecule, with fewer steric restrictions.

DISCUSSION

The existence of several conjugated pyridinoproteins has been postulated by HUENNEKENS AND GREEN[15,16], HUENNEKENS[17] and MAHLER *et al.*[18]. As a matter of fact most of the pyridine nucleotides probably exist in the protein-bound form within the mitochondrion, and are not freely diffusible. The question has frequently been raised how such pyridinoproteins could interact with each other and with the enzymes of electron transport. It is believed that the experiments of CORI, VELICK AND CORI[2], as well as the present ones, at least indicate the feasibility of direct interaction between DPNH bound to enzyme proteins at a catalytically active site and other enzymes, without the intervention of freely diffusible pyridine nucleotide in the medium. The feasibility of such reactions, involving, in the present case, the interaction at a sterically well-defined site of three proteins of molecular weight 120,000, 80,000 and 16,000 (GPD, reductase and cytochrome *c* respectively) also raises interesting implications from the point of view of kinetics and mechanism of enzymic reactions.

ACKNOWLEDGEMENTS

The present investigation had its origin in a discussion with Prof. PAUL BOYER of the Department of Agricultural Biochemistry, University of Minnesota, of his proposed mechanism of GPD action. We are also greatly indebted to him for providing us with GPD. We also wish to acknowledge many useful discussions with Drs. D. E. GREEN, D. RAO SANADI, T. P. SINGER and E. KEARNEY.

SUMMARY

The interaction of GPD-bound DPNH with cytochrome *c* or 2,6-dichlorophenolindophenol in the presence of DPNH-cytochrome reductase has been demonstrated. Two types of reaction have been observed, a stoichiometric one where both initial rate and total extent of reaction are functions of the concentration of GPD added, and a catalytic one in the presence of arsenate, which leads to an extensive reduction of the oxidant with a linear rate also dependent on GPD concentration. The reduction rates are of an order of magnitude comparable to that with free DPNH under similar experimental conditions, thus excluding the possibility of their being due to the small dissociation of the DPNH-enzyme complex. The stoichiometry of the reaction with cytochrome and indophenol is such that only one of the two bound DPNH molecules appears to be capable of reducing cytochrome, while both are active in the indophenol reaction. The implication of these findings for other conjugated pyridinoproteins, notably within the mitochondrial structure, are discussed.

References p. 107.

RÉSUMÉ

Les auteurs ont démontré l'interaction entre le DPNH lié au GPD avec le cytochrome *c* ou le 2,6-dichlorophénolindophénol en présence de la DPNH-cytochrome réductase. Deux types de réactions s'observent, l'une stoechiométrique, dans laquelle la vitesse initiale et l'importance finale de la réaction sont des fonctions de la concentration du GPD ajouté, l'autre catalytique en présence d'arsénate, qui entraîne une réduction importante de l'oxydant dont la vitesse dépend aussi linéairement de la concentration en GPD. Les vitesses de réduction sont d'un ordre de grandeur comparable à celles qu'on observe avec le DPNH libre dans les mêmes conditions expérimentales, ce qui exclut que la réduction puisse être due à la faible dissociation du complexe DPNH-enzyme. La stoechiométrie de la réaction avec le cytochrome et l'indophénol est telle qu'une seule des molécules de DPNH liées semble capable de réduire le cytochrome, tandis que les deux sont actives vis à vis de l'indophénol. La signification de ces résultats pour d'autres pyridinoprotéines conjuguées, en particulier celles des mitochondries, est discutée.

ZUSAMMENFASSUNG

Die Reaktion von GPD-gebundenen DPNH mit Cytochrom *c* oder 2.6-Dichlorphenolindophenol in Gegenwart von DPNH-Cytochrom-Reduktase wurde gezeigt. Es wurden 2 Reaktionstypen beobachtet: eine stöchiometrische, bei der sowohl die Anfangsgeschwindigkeit wie das totale Ausmass der Reaktion Funktionen der Konzentration der hinzugefügten GPD sind und eine katalytische in Gegenwart von Arsenat, die zu einer weitgehenden Reduktion des Oxydationsmittels mit einer linearen, ebenfalls von der GPD-Konzentration abhängigen Geschwindigkeit führt. Die Reduktionsgeschwindigkeiten sind von einer Grössenordnung, die vergleichbar ist mit der freier DPNH unter ähnlichen experimentellen Bedingungen und so die Möglichkeit ausschliesst, dass sie einer geringen Dissoziation des DPNH-Enzymkomplexes zuzuschreiben sind. Die Stöchiometrie der Reaktion mit Cytochrom und Indophenol ergibt, dass scheinbar nur eine der zwei gebundenen DPNH-Moleküle fähig ist Cytochrom zu reduzieren, während bei der Reaktion mit Indophenol beide aktiv sind. Die Folgerung dieser Entdeckungen für andere konjugierte, innerhalb der Mitochondrienstruktur zu bemerkende Pyridinoproteine werden besprochen.

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