THE COMBINATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE WITH GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE

by

CARL F. CORI, SIDNEY F. VELICK, AND GERTY T. CORI

Department of Biological Chemistry, Washington University, School of Medicine, St. Louis, Missouri (U.S.A.)

It has been shown in a previous paper[1] that glyceraldehyde phosphate dehydrogenase from rabbit muscle contains one mole of diphosphopyridine nucleotide, (DPN) per 50000 g of protein. This ratio did not change after prolonged dialysis against distilled water or after repeated recrystallizations from ammonium sulphate solutions. When an aqueous solution of the enzyme was treated with activated charcoal (norit) and filtered, DPN was removed. Addition of an excess of DPN and ammonium sulphate to the DPN-free enzyme solution resulted in the formation of crystals which contained the original ratio of DPN to protein. From these and other observations it was concluded that the enzyme contained firmly bound DPN. The fact that DPN could be removed with norit made it clear that the union between enzyme and coenzyme was not through a covalent bond.

Earlier work[2] had indicated that the dissociation constant of the enzyme with DPN, as estimated from the concentration of DPN at which the reaction with glyceraldehyde phosphate occurred at half maximal velocity, was of the order of $4 \cdot 10^{-8}$ M/ml. This agreed with a value obtained by WARBURG AND CHRISTIAN[3] with yeast enzyme and free glyceraldehyde as substrate. According to existing criteria the constant so obtained is sufficiently large to permit easy separation of enzyme and coenzyme by dialysis or recrystallization. The fact that such a separation was not observed suggests either that the enzyme combines with DPN at two sites, one of which binds DPN more firmly than the other, or that the conclusions drawn from the kinetic measurements or from dialysis and recrystallization are not valid.

In the present paper experiments are described in which some aspects of the two-site hypothesis are tested. In order to make reactions of bound DPN measurable in a 1 cm cell at 340 mμ in the Beckman spectrophotometer, it is necessary to use enzyme concentrations of 2 to 4 mg per ml which are about 1000 times greater than those necessary to give good rates with added DPN and glyceraldehyde phosphate. Accordingly the reaction with glyceraldehyde phosphate is too rapid for convenient study, unless one works at a p_H far from the optimum. When glyceraldehyde is used as substrate, however, the reaction rate is conveniently measurable over a wide range of conditions, the slower reaction being due, as will be shown, to a low affinity of glyceraldehyde for the enzyme.

References p. 169.

EXPERIMENTAL

The enzyme was prepared as previously described[2] and recrystallized four times. An aliquot of the crystal suspension in ammonium sulphate was centrifuged at about 10000 rpm, drained, and dissolved in 0.03 M sodium pyrophosphate — 0.003 M cysteine buffer at p_H 8.3. This enzyme solution was prepared fresh for each experiment. The composition of reaction mixtures is given in the tables.

THE DISSOCIATION CONSTANT OF ENZYME AND BOUND DPN

The enzyme and bound DPN concentrations cannot be varied independently unless one resorts to partial removal of DPN with norit. The latter procedure introduces additional variables due to the instability of the DPN-free enzyme and so a dilution method was employed. It was possible to follow the reactions in the more dilute solutions by using cuvettes with a longer light path.

The experiment consisted in comparing the rates of reaction in two solutions identical in all concentrations except that of the enzyme-DPN complex. The results of such an experiment are described in Table I. It may be seen that the directly measured

TABLE I

THE DISSOCIATION OF ENZYME AND "BOUND" DPN

Two reaction mixtures were prepared, one with a total volume of 6 ml and the other of 30 ml. The former was in a cell of 2 cm and the latter in a cell of 10 cm length. Both reaction mixtures contained in moles per ml, $6 \cdot 10^{-6}$ arsenate, $3 \cdot 10^{-6}$ cysteine, $5 \cdot 10^{-5}$ pyrophosphate (p_H 8) and $2 \cdot 10^{-6}$ DL-glyceraldehyde (the latter added to start the reaction). The two reaction mixtures differed however in that the 2 cm cell contained $1.77 \cdot 10^{-8}$ and the 10 cm cell $3.54 \cdot 10^{-9}$ M per ml of enzyme – DPN.

Time (min)	2 cm cell $\log I_0/I$	10 cm cell $\log I_0/I$
1	0.022	0.019
2	0.037	0.037
3	0.053	0.053
4	0.067	0.067
5	0.079	0.081
6	0.091	0.092
7	0.099	0.101
∞*	0.223	0.222

* After addition of glyceraldehyde phosphate.

rates were identical. This means that the decrease in rate due to the 5-fold dilution of enzyme-DPN complex was exactly compensated by the 5-fold increase in light path. Since the observed rate was proportional to the concentration of undissociated enzyme-DPN, it follows that no measurable increase in dissociation occurred on dilution. In order for this condition to hold, it would be necessary for the dissociation constant of enzyme-DPN to be of the order of $1 \cdot 10^{-10}$ M/ml or less. Since in fact no evidence of dissociation was obtained at all in this experiment, the above figure may be considered only to be an upper limit*. An analogous dilution experiment with a small amount of enzyme and added DPN with glyceraldehyde phosphate as substrate showed a change

* In work which will be reported in detail at a later date it has been shown that bound DPN equilibrates rapidly with radioactive DPN labelled with P^{32}. This is in harmony with the conclusion that the bond between DPN and enzyme is not of the covalent type and that the bound DPN exhibits a finite dissociation.

References p. 169.

in rate between DPN concentrations of $4.4 \cdot 10^{-8}$ and $4.4 \cdot 10^{-9}$ M/ml that is consistent with a dissociation constant of the order of $4 \cdot 10^{-8}$ M/ml.

The fact that depending upon whether or not one measures bound DPN or added DPN, one gets apparent dissociation constants differing by a factor of *at least* 100 argues for the existence of two types of catalytic sites. We will designate the still hypothetical site with the higher DPN affinity as site I and the site with lower DPN affinity as site II and proceed to examine the conditions that would hold during the course of a reaction.

THE REACTION AT SITE I

In Table II is shown an experiment in which the reduction of bound DPN is studied as a function of glyceraldehyde concentration. The glyceraldehyde concentration in all

TABLE II

EFFECT OF CONCENTRATION OF GLYCERALDEHYDE

Reaction mixture consisted (in moles per ml) of $2.4 \cdot 10^{-8}$ enzyme – DPN, $6 \cdot 10^{-6}$ arsenate, $3 \cdot 10^{-6}$ cysteine, $5 \cdot 10^{-5}$ pyrophosphate (p_H 8.3) and varying amounts of DL-glyceraldehyde.

Time in min	Concentration of glyceraldehyde (as D-form, moles per ml)					
	$0.5 \cdot 10^{-6}$		$1 \cdot 10^{-6}$		$2 \cdot 10^{-6}$	
	$\log I_0/I$	K*	$\log I_0/I$	K*	$\log I_0/I$	K*
1.5	0.028	0.14	0.051	0.27	0.087	0.57
3.0	0.054	0.15	0.088	0.29	0.123	0.55
4.5	0.072	0.14	0.109	0.29	0.140	0.57
6.0	0.085	0.14	0.123	0.28	0.147	0.57
7.5	0.096	0.13	0.130	0.26	0.150	
9.0	0.103	0.13	0.136		0.152	
10.5	0.108		0.140		0.152	
		0.14		0.28		0.56

* $K = 2.3/t \log A\ (A - x)$, A = initial concentration of DPN.

cases was sufficiently higher than that of DPN so that it was virtually constant during the course of the reaction. Under these conditions the rate is described by a first order velocity constant. The fact that the first order constants increase linearly with initial glyceraldehyde concentration means that saturation of the enzyme with glyceraldehyde has not been approached. The dissociation constant of enzyme-glyceraldehyde is therefore very large.

At the concentrations of enzyme employed the amount of free DPN in equilibrium with the protein would be negligible if the dissociation constant at site I is less than $1 \cdot 10^{-10}$. The above reaction is therefore first order with respect to enzyme-DPN complex. This means that each enzyme molecule behaves as though it reacted only once.

When DPNH (in amounts equivalent to the bound DPN present) was added at the beginning of the reaction, it exerted an inhibitory effect. This is indirect evidence that DPNH as well as DPN is bound at site I. It is also possible to demonstrate in a direct manner that DPNH is bound. This was done by reducing the bound DPN in a solution containing 10 to 20 mg of enzyme per ml with excess glyceraldehyde phosphate and

References p. 169.

arsenate and then precipitating the enzyme with ammonium sulphate at a final concentration of 85% saturation. It was found that 90% or more of the enzyme was precipitated and that the ratio of DPNH to protein in the precipitate was the same as that of DPN to protein in the original solution.

For the interpretation of reactions with added DPN an additional consideration is important, namely, whether added DPN can displace DPNH at site I. From the fact that DPN at site I is dissociable one would expect the same to hold for DPNH. The problem of displacement would then be resolved by a determination of the relative dissociation constants of enzyme with DPN and DPNH. Theoretically this could be done by determining the ratio of DPN to DPNH in the enzyme when enzyme-DPNH is precipitated in the presence of added DPN.

A preliminary experiment of this type is presented in Table III; it gives qualitative evidence that displacement of DPNH by DPN does occur and that the dissociation

TABLE III

COMPETITION BETWEEN DPN AND DPNH

DPN in enzyme was reduced by addition of arsenate and an equivalent amount of triosephosphate. Aliquots of the reduced enzyme were treated as follows. In (A) 0.5 ml of enzyme containing 12.5 mg of protein, + 0.1 ml of H_2O, was precipitated with 3 ml of saturated ammonium sulphate. In (B) 0.5 ml of enzyme + 0.1 ml of DPN solution ($2.4 \cdot 10^{-7}$ M) was incubated for 3 minutes before being precipitated with ammonium sulphate. The precipitates were separated by centrifugation at 10000 rpm and dissolved in cysteine-pyrophosphate buffer.

	A				B			
	Vol. in ml	Protein mg	D_{340}	DPNH $M \cdot 10^7$	Vol. in ml	Protein mg	D_{340}	DPNH $M \cdot 10^7$
Supernatant fluid	3.6	1.3*	0.063	0.36	3.6		0.275	1.57
Precipitate	3.1	11.3**	0.304	(1.49)	3.1	11.1**	0.175	0.86
Pptate + $HAsO_4^=$ + triosephosph.***	3.25		0.413	2.13				
				2.49				2.43

* Calculated from optical density at 276 mμ.
** Determined by biuret method.
*** An excess of glyceraldehyde phosphate was added in order to reduce DPN completely.

constants of the oxidized and reduced forms with the enzyme are at least of the same order of magnitude. The chief objection that might be raised is that the high concentration of ammonium sulphate may change the equilibrium.

An analysis of the experiment shows that although a stoichiometric amount of glyceraldehyde phosphate was used, the reaction was only 70% complete when DPN was added. This value is calculated from the additional DPNH which appeared when excess triosephosphate and arsenate was added to the dissolved precipitate of the enzyme in experiment A. Accordingly there must have been residual triosephosphate in B when DPN was added. The excess DPN in B then drove the reaction to completion as shown by the DPNH recoveries in A and B.

Some of the DPNH in the supernatant fluid of B, therefore, arose by reduction of added DPN and hence did not represent DPNH displaced from the enzyme. A rough estimate of the amount actually displaced is (by comparison with experiments A) equal

References p. 169.

to the total amount in the supernatant of B ($1.57 \cdot 10^{-7}$ M) minus the amount arising from residual triosephosphate, [$(2.13 - 1.49) \cdot 10^{-7} = 0.64 \cdot 10^{-7}$], minus unprecipitated protein-DPNH ($0.36 \cdot 10^{-7}$). The net displaced DPNH is $(1.57 - 0.64 - 0.36) \cdot 10^{-7} = 0.57 \cdot 10^{-7}$ M. A similar value is arrived at by comparing DPNH in the precipitated protein in A and B, namely $(1.49 - 0.86) \cdot 10^{-7} = 0.63 \cdot 10^{-7}$ M.

THE REACTION AT SITE II

When reactions are studied with added DPN, site I is saturated, even at low enzyme concentrations and site II is saturated to an extent which depends upon its dissociation constant and the concentration of free DPN. Reaction will be expected to occur at both sites but the DPNH formed at site I will be displaced by DPN in solution and site I as well as site II will now have a "turnover". The reactions at both sites will be first order provided that at each site the affinity for DPN is the same as that for DPNH. Experimentally it was found that the rate remained first order when DPN was added, Table IV.

TABLE IV

EFFECT OF ADDED DPN ON RATE OF REACTION

The enzyme concentration corresponded to $3.4 \cdot 10^{-8}$ M of bound DPN per ml, the p_H was 8.3 and the temperature 26°. No DPN was added in A, while in B and C, 3.4 and $7 \cdot 10^{-8}$ M per ml respectively was added, giving the total of concentrations of DPN shown in the table headings. The reaction was started by the addition of glyceraldehyde (final concentration as the D-form $1.1 \cdot 10^{-6}$ M per ml). $K = 2.3/t \log A/(A - x)$, A being the initial concentration of DPN. V_0 (initial velocity) = K times the initial concentration of DPN.

Time in min	A		B		C	
	$3.4 \cdot 10^{-8}$ M/ml		$6.8 \cdot 10^{-8}$ M/ml		$10.4 \cdot 10^{-8}$ M/ml	
	$\log I_0/I$	K	$\log I_0/I$	K	$\log I_0/I$	K
1.5	0.066	0.25	0.105	0.19	0.112	0.12
3.0	0.111	0.24	0.191	0.20	0.217	0.13
4.5	0.142	0.24	0.248	0.19	0.302	0.14
6.0	0.160	0.23	0.290	0.19	0.371	0.14
7.5	0.172	0.22	0.320	0.18	0.426	0.14
9.0	0.183	0.22	0.343	0.18	0.468	0.14
∞*	0.214	—	0.428	—	0.658	—
		0.233		0.188		0.136
V_0		0.79		1.28		1.41

* After addition of glyceraldehyde phosphate.

By multiplying first order velocity constants, K, by the initial concentrations of DPN one gets the initial velocity of the reaction, V_0, in terms of M. min^{-1} ml^{-1}. The observed increase in initial rate on addition of DPN can be seen to be approaching a maximum value which would correspond to the saturation of both sites with DPN. Because of the high concentration of enzyme, one cannot calculate the enzyme-coenzyme dissociation constants by the usual methods (which are based on the assumption that the concentration of free DPN is not appreciably diminished by combination with the enzyme). It is furthermore not possible from this experiment to reach unambiguous conclusions with respect to the number and type of catalytic sites.

References p. 169.

EQUILIBRIUM CONSTANTS

The equilibria of reactions with free DPN and substrate using catalytic amounts of enzyme and of reactions between bound DPN and substrate with the protein present in quantities equivalent to the DPN may be formulated respectively as follows:

a) DPN + 3-glyceraldehyde phosphate + $HPO_4^=$ $\overset{\text{enzyme}}{\rightleftharpoons}$ DPNH + H^+ + 1,3-diphosphoglyceric acid

b) DPN-enzyme + 3-glyceraldehyde phosphate + $HPO_4^=$ $\rightleftharpoons$ DPNH-enzyme + H^+ + 1,3-diphosphoglyceric acid.

In the former case which is a true catalytic reaction, the enzyme forms transient intermediates with a minute fraction of the substrate at any given time. Case (b) is in effect a different reaction in which not free DPN and DPNH but the corresponding protein complexes are reactants.

MEYERHOF AND OESPER[4] have carried out a detailed study of the reaction as represented by (a). Since one hydrogen ion enters the equilibrium, the equilibrium constant showed a dependence upon p_H. Equilibrium measurements were made with added DPN under conditions similar to those employed by MEYERHOF AND OESPER. About 30 γ of enzyme per ml were used so that equilibrium was reached within one minute after addition of glyceraldehyde phosphate, even at low p_H values. Concentrations of DPN and glyceraldehyde phosphate in the stock solutions were determined optically by enzymatic methods. p_H was measured with a glass electrode in the reaction mixture at the end of the experiment. The values found for the equilibrium constants fall well within the range reported by MEYERHOF AND OESPER, Table V.

TABLE V

EQUILIBRIUM OF REACTION AT DIFFERENT p_H

The equilibrium is compared for catalytic amounts of enzyme (C) plus added DPN, and large amounts of enzyme (L) containing bound DPN. The initial and final concentrations are given in moles per liter. GAP = glyceraldehyde phosphate.

Amount of enzyme	Initial Concentrations			Final Concentrations			Present data		Data of M. and O.[4]	
	DPN	GAP	PO_4	DPN	GAP	PO_4	K	p_H	K	p_H
	$\cdot 10^5$	$\cdot 10^3$	$\cdot 10^3$	$\cdot 10^5$	$\cdot 10^3$	$\cdot 10^3$				
L	6.23	1.43	82.8	4.08	1.39	82.8	0.67	7.09		
C	7.48	1.42	82.8	4.62	1.37	82.8	0.65	7.08	0.6–1.4	7.15
L	5.46	1.43	8.66	4.45	1.38	8.62	16.4	7.85	19.8*	7.85
C	7.53	1.43	8.66	6.35	1.36	8.60	28.9	8.10	21–28	8.20

* Calculated from MEYERHOF AND OESPER'S[4] data by means of their complete equilibrium equation.

For equilibrium measurements under the conditions of case (b) two parallel reaction mixtures were prepared which differed only in that one contained phosphate and the other arsenate. The former was used for equilibrium determination while the latter served for determination of the amount of DPN present in the enzyme. The value of the equilibrium constants that were obtained agree within experimental limits with those found with small amounts of enzyme and added DPN.

Although one cannot derive from these measurements evidence for the existence

References p. 169.

of two catalytic sites, the following considerations are of interest. In case (a) the enzyme cannot contribute to the net free energy change which is fixed by the initial and final states of the free reactants. In case (b) two of the reactants have been altered by complex formation and the initial and final energy states are not the same as in case (a). However, since only the *difference* in initial and final states determines the net free energy change, case (b) may or may not have the same equilibrium constant as case (a). These considerations apply irrespective of the physical nature of the bonding forces involved and the number and type of binding sites.

It may be inferred from the kinetics that the protein has the same affinity for DPN as for DPNH*. Conclusions concerning the relative dissociation constants of enzyme-DPN and enzyme-DPNH may also be drawn from a comparison of the equilibrium constants in (a) and (b). If the binding of the other reactants does not alter their energy differences then, from the equality of equilibrium constants, it follows that the dissociation constants of enzyme-DPN and enzyme-DPNH are equal.

p_H OPTIMUM

The rate of the reaction of glyceraldehyde with enzyme DPN was measured at p_H 8.4, 7.5, and 6.4 in cysteine-pyrophosphate buffer. The relative rates calculated from the first order velocity constants were as 100:30:9. This agrees with the p_H activity curve as determined previously with small amounts of enzyme (6 γ/ml) and addition of DPN and glyceraldehyde phosphate as substrate[2].

REACTION WITH LACTIC DEHYDROGENASE

It has been shown in a previous report[1] that enzyme DPN, after reduction by glyceraldehyde phosphate, was reoxidized by addition of sodium pyruvate and a purified preparation of lactic dehydrogenase from rabbit muscle. The simplest explanation of this result is that the bound DPNH has a small but finite tendency to dissociate and that it is the dissociated DPNH which reacts with the pyruvate-lactic dehydrogenase system. In these experiments lactic dehydrogenase was present in considerable excess, so that the rate of the reaction could not be measured.

The dissociation constant for lactic dehydrogenase and DPNH has been determined by KUBOWITZ AND OTT[5] who report a value of $5 \cdot 10^{-9}$ M/ml. In experiment A, Table VI, the initial concentration of bound DPNH was $\frac{2.3 \cdot 0.146}{1.45 \cdot 10^{7}} = 2.3 \cdot 10^{-8}$ M/ml. If the DPNH-enzyme dissociation constant were $1 \cdot 10^{-10}$ M/ml, there would not be enough free DPNH in solution to give 25% saturation of lactic dehydrogenase and the rate of reaction would be much slower than in experiment C, where the concentration of added DPNH was $3.1 \cdot 10^{-8}$M/ml or enough to saturate the enzyme. The fact that such a difference**

* This inference arises from the fact that in the presence of a large excess of glyceraldehyde and arsenate the reduction of bound and of added DPN may be described by a first order velocity constant. If one assumes that DPNH has the same affinity for the catalytic site as does DPN, then the first order kinetics may be shown to be due to the formation of DPNH which acts as a competitive inhibitor[3].

** Actually the rate was faster in A than in C. One possible explanation was that lactic dehydrogenase in C was acting in the absence of "protective" protein. In order to compensate for this difference, lactic dehydrogenase was added in other experiments to a solution containing the same amount of triosephosphate dehydrogenase the DPN of which had not been reduced. The rate of reaction of lactic dehydrogenase with "bound" and with added DPNH was then approximately the same.

References p. 169.

TABLE VI

REACTION OF "BOUND" DPNH WITH LACTIC DEHYDROGENASE SYSTEM

The DPN in 24 mg of glyceraldehyde phosphate dehydrogenase was first reduced by addition of glyceraldehyde phosphate and arsenate. One aliquot (A) was precipitated directly with ammonium sulphate, while another aliquot (B) was first exposed to 0.024 M iodoacetate before being precipitated with ammonium sulphate. The precipitates were separated by centrifugation, dissolved in cysteine-pyrophosphate buffer, p_H 8.3, and pyruvate ($1 \cdot 10^{-5}$ M/ml) was added. The reaction was started by the addition of a catalytic amount of lactic dehydrogenase. To reaction mixture (C) free DPNH was added in place of glyceraldehyde phosphate dehydrogenase containing bound DPNH.

Time in min	A		B		C	
	log I_0/I	Δ	log I_0/I	Δ	log I_0/I	Δ
0	0.146		0.146		0.195	
1	0.112	0.034	0.098	0.048	0.169	0.026
2	0.084	0.062	0.071	0.075	0.153	0.042
3	0.068	0.078	0.050	0.096	0.140	0.055
4	0.050	0.096	0.037	0.109	0.127	0.068
5	0.045	0.101	0.029	0.117	0.113	0.082

in rate was not observed leaves one with two alternatives. Either DPNH is more highly dissociated than has been assumed or lactic dehydrogenase can react with bound DPNH. The latter alternative would involve collisions between protein molecules which, from a kinetic standpoint, is not incompatible with a rapid rate of reaction.

In a final experiment we tried to see whether the lactic dehydrogenase system could reduce the DPN bound to the enzyme. The reaction mixture contained 4 mg of glyceraldehyde phosphate dehydrogenase per ml as a source of DPN, sodium lactate, cyanide (to trap the pyruvate formed) and a catalytic amount of lactic dehydrogenase. The bound DPN was reduced at a good rate as soon as the lactic dehydrogenase was added. The considerations mentioned above when the reverse reaction was discussed apply here as well.

IODOACETATE

Iodoacetate (0.004 M) completely inhibited the reduction of enzyme DPN by substrate. An enzyme solution of about 8 mg of protein per ml was prepared with pyrophosphate buffer at p_H 8.4 containing no cysteine. Five minutes at 25° was allowed for reaction with iodoacetate before glyceraldehyde was added. A suitable control without iodoacetate was run simultaneously. This was necessary because the enzyme loses activity quite rapidly in the absence of cysteine. No enzymatic activity could be detected in the presence of iodoacetate. Whether or not a differentiation of two catalytic sites is possible by means of addition of smaller concentrations of iodiacetate has not been tried.

In experiment B, Table V, iodoacetate was added after the DPN bound to the enzyme had been reduced. The object was to see whether the inactivation of the enzyme by iodoacetate would influence the rate of reaction of bound DPNH with the lactic dehydrogenase system. As shown in Table V no difference could be detected.

This paper is presented as a token of esteem for the numerous scientific contributions of OTTO MEYERHOF.

References p. 169.

SUMMARY

The theory has been examined that glyceraldehyde phosphate dehydrogenase from rabbit muscle contains two catalytic sites, having dissociation constants with DPN which differ by a factor of 100 or more. The facts in favour of a very slightly dissociated site are that the enzyme retains on recrystallization or dialysis a stoichiometric amount of DPN. From observations made in kinetic measurements this DPN does not measurably dissociate on five fold dilution of the enzyme. Furthermore, evidence is presented that DPNH is also bound to the enzyme and that it can be displaced by added DPN to an extent which indicates relative affinities of the protein for the oxidized and reduced forms of at least the same order of magnitude. The fact that bound DPN can be removed from the enzyme by adsorption on charcoal and that it exchanges rapidly with DPN labelled with P^{32} allows the conclusion (a) that the binding is not of the covalent type and (b) that bound DPN has a measurable dissociation.

Other approaches to the problem did not reveal differences between the reaction with enzyme-DPN and the reaction with a catalytic amount of enzyme plus added DPN. In both cases, in the presence of an excess of substrate, the reaction was first order with respect to the total DPN concentration, and the p_H optimum was the same. The equilibrium constants with bound and with added DPN were also the same. Iodoacetate inhibited the reaction at the bound site. Kinetic studies involving simultaneous reaction of bound and added DPN showed that with increasing concentrations of the latter a saturation value was approached, but the data could not be resolved to give an unequivocal answer in terms of two catalytic sites.

Enzyme DPNH was shown to react rapidly with lactic dehydrogenase plus pyruvate, or in the reverse reaction, bound DPN was found to react with lactic dehydrogenase plus lactate. On the basis of the assumption that bound DPNH has a very low dissociation, the observed rate of reaction with lactic dehydrogenase would have to be attributed to collisions between protein molecules.

In the light of available evidence the hypothesis that glyceraldehyde phosphate dehydrogenase has two catalytic sites which differ in their affinity for DPN requires further examination.

RÉSUMÉ

Un examen a été fait de la théorie selon laquelle la déshydrogénase de l'aldéhyde phosphoglycérique du muscle de lapin posséderait deux positions catalytiques dont les constantes de dissociation avec le DPN différeraient par un facteur de 100 ou davantage. Les faits en faveur d'une position où la dissociation est très faible sont que l'enzyme, lors de la recristallisation ou de la dialyse, retient une quantité stoéchiométrique de DPN. D'observations faites au cours de mesures cinétiques, il découle que ce DPN ne dissocie pas d'une façon appréciable lorsqu'on dilue l'enzyme au cinquième. En outre, des preuves sont apportées que le DPNH est lui aussi lié à l'enzyme et peut être déplacé de cette combinaison par l'addition de DPN, jusqu'à une limite qui indique que les affinités relatives de la protéine pour la forme oxydée et pour la forme réduite sont en tout cas du même ordre de grandeur. Le fait que le DPN lié peut être éliminé de l'enzyme par adsorption à du charbon actif, et qu'il s'établit un échange rapide avec du DPN marqué au P^{32}, permet de conclure: a) que le mode de liaison n'est pas du type covalent et b) que le DPN possède une dissociation mesurable.

D'autres méthodes d'approche du problème posé n'ont pas révélé de différences entre la réaction de la combinaison enzyme-DPN et celle d'une quantité catalytique d'enzyme plus du DPN additionné. Dans les deux cas, en présence d'un excès de substratum, la réaction était du premier ordre par rapport à la concentration totale en DPN, et le p_H optimum était le même. Les constantes d'équilibre avec du DPN lié ou additionné étaient également identiques. L'acide iodacétique inhibe la réaction au point de liaison. Des études cinétiques impliquant la réaction simultanée de DPN lié et de DPN additionné ont montré que lorsque les concentrations de ce dernier augmentent, on tend vers une valeur de saturation, mais il n'a pas été possible d'ordonner les résultats de façon à donner une réponse non équivoque à la question de l'existence de deux positions catalytiques.

Il a été montré que le DPNH lié réagit rapidement avec la déshydrogénase lactique plus pyruvate, ou, en sens inverse, le DPN lié avec la déshydrogénase lactique + lactate. Si l'on assume que le DPNH lié dissocie très faiblement, la vitesse observée de la réaction avec la déshydrogénase lactique devrait être attribuée à des collisions entre des molécules de protéine ou à la formation de complexes enzymatiques organisés. A la lumière des faits établis, l'hypothèse que la déshydrogénase de l'aldéhyde phospho-glycérique possède deux positions catalytiques différant par leur affinité pour le DPN demande de nouvelles études.

ZUSAMMENFASSUNG

Es wurde die Theorie untersucht, welche besagt dass Glycerinaldehydphosphat-Dehydrogenase aus Kaninchenmuskel zwei katalytische Stellen besitzt, deren Dissoziationskonstanten mit DPN um

References p. 169.

mehr als das Hundertfache von einander abweichen. Die Tatsachen, die für eine sehr wenig dissoziierte Haftstelle sprechen, sind die, dass das Enzym beim Umkristallisieren oder bei der Dialyse eine stöchiometrische Menge DPN zurückhält. Aus Beobachtungen bei kinetischen Messungen geht hervor, dass dieses DPN bei fünffacher Verdünnung des Enzyms nicht messbar dissoziiert. Obendrein werden Belege dafür erbracht, dass auch DPNH an das Enzym gebunden ist, und aus dieser Verbindung durch zugesetztes DPN verdrängt werden kann bis zu einem Grade, welcher relative Affinitäten des Proteins zur oxydierten und zur reduzierten Form von mindestens gleicher Grössenordnung anzeigt. Die Tatsache, dass gebundenes DPN durch Adsorption an Kohle aus dem Enzym entfernt werden kann, und dass die Austauschreaktion mit DPN welches mit P^{32} markiert ist eine rasche ist, erlaubt den Schluss: a) dass die Bindung nicht covalenter Art ist und b) dass gebundenes DPN messbar dissoziiert.

Andere Angriffsarten auf das gestellte Problem zeigten keine Unterschiede auf zwischen der Reaktion mit Enzym-DPN und der Reaktion mit einer katalytischen Menge Enzym plus zugesetztem DPN. In beiden Fällen war, in Gegenwart eines Überschusses an Substrat, die Reaktion erster Ordnung mit Bezug auf die gesamte DPN-Konzentration und das p_H-Optimum war das Gleiche. Die Gleichgewichtskonstante mit gebundenem und mit zugesetztem DPN war ebenfalls dieselbe. Jodacetat hinderte die Reaktion an der Bindungsstelle. Kinetische Untersuchungen, bei welchen gleichzeitig gebundenes und zugesetztes DPN reagierte, zeigten an, dass man sich mit wachsender Konzentration des Letzteren einem Sättigungswert näherte; jedoch konnten die Ergebnisse nicht so dargestellt werden, dass sie eine unzweideutige Antwort auf die Frage gegeben hätten, ob zwei katalytische Stellen bestehen.

Es wurde gezeigt, dass gebundenes DPNH rasch mit Milchsäure-Dehydrogenase plus Pyruvat reagierte, oder in umgekehrter Richtung gebundenes DPN mit Milchsäure-Dehydrogenase plus Lactat. Auf der Grundlage der Annahme, dass gebundenes DPNH sehr wenig dissoziiert, müsste die beobachtete Reaktionsgeschwindigkeit mit Milchsäure-Dehydrogenase durch Zusammenstösse zwischen Proteinmolekeln erklärt werden, oder durch die Bildung von geordneten Enzym-Komplexen. Im Lichte der vorhandenen Belege gesehen bedarf die Hypothese, dass Glycerinaldehydphosphat-Dehydrogenase zwei katalytische Stellen besitzt, welche sich in ihrer Affinität für DPN unterscheiden, weiterer Untersuchung.

REFERENCES

1 J. F. TAYLOR, S. F. VELICK, G. T. CORI, C. F. CORI, AND M. W. SLEIN, *J. Biol. Chem.*, 173 (1948) 619.
2 G. T. CORI, M. W. SLEIN, AND C. F. CORI, *J. Biol. Chem.*, 173 (1948) 605.
3 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 303 (1939) 40.
4 O. MEYERHOF AND P. OESPER, *J. Biol. Chem.*, 170 (1947) 1.
5 F. KUBOWITZ AND P. OTT, *Biochem. Z.*, 314 (1943) 94.

Received April 19th, 1949