The Structure of the Abortive Diphosphopyridine Nucleotide-Pyruvate-Lactate Dehydrogenase Ternary Complex as Determined by Proton Magnetic Resonance Analysis*

(Received for publication, July 17, 1973)

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SUMMARY

The ternary complex, consisting of DPN⁺, pyruvate, and chicken heart lactate dehydrogenase, can be dissociated in base to yield a stable cyclic binary adduct. This DPN⁺-pyruvate adduct, which is a dithydropyridine derivative, is identical in structure with one of the two diastereomers produced chemically by the addition of pyruvate to DPN⁺ in basic solution. A structural analysis of this enzyme-generated adduct by proton magnetic resonance has led to the following conclusions concerning the 322-nm-absorbing ternary complex of DPN⁺, enolpyruvate, and the lactate dehydrogenases: (a) a covalent bond forms between DPN⁺ and enolpyruvate; (b) the substitution of pyruvate on DPN⁺ occurs at the nicotinamide C₁ position of DPN⁺; (c) the substitution takes place on the A side of the nicotinamide ring; and (d) this pyridine C₆-substituted adduct of DPN⁺ is capable of dissociating intact from lactate dehydrogenase.

The strong inhibition of lactate dehydrogenase which occurs at elevated pyruvate concentrations has been attributed to an abortive ternary complex of DPN⁺, pyruvate, and the enzyme (1-5). This complex possesses an absorption maximum at 322 nm and causes a significant quenching of protein fluorescence concomitant with its formation (6). There is strong evidence that the tautomeric enol form of pyruvate is responsible for this inhibition (7, 8) and not the keto form, since no inhibition occurs when only the keto form of pyruvate is present (7). Consistent with this result are recent studies which show that the keto tautomer is the "active" form of pyruvate in the enzymatic reaction (9).

We, as well as others, have found that the lactate dehydrogenases isolated from heart muscle are more prone to this substrate inhibition than those isolated from skeletal muscle (10-15). We have also postulated that the differences in sensitivity toward substrate inhibition than those isolated from skeletal muscle (10-15).

EXPERIMENTAL PROCEDURE

Chicken heart lactate dehydrogenase was prepared and assayed as reported previously (20). The DPN-pyruvate base-catalyzed adduct was prepared as reported by Everse et al. (17) and purified by eluting it from a DEAE-11 column in the bicarbonate form with a linear 0 to 0.5 M gradient of NH₄HCO₃. The DPN-pyruvate adduct eluted at approximately 0.35 M NH₄HCO₃.

The DPN-pyruvate adduct synthesized with the chicken heart lactate dehydrogenase was produced by incubating 300 mg of DPN⁺ (420 pmol) with 300 mg of pyruvate (27 mmol), and 220 × 10⁶ units (200 mg) of ammonium sulfate-free chicken heart lactate dehydrogenase in 300 ml of 0.05 M Tris and adjusting the pH to 10.0. The reaction was followed by monitoring the increase in 340-nm absorption with time. When an optical density of 0.5 had been obtained the reaction was stopped by lowering the pH to 8.0 with concentrated HCl. After pressure dialysis through an Amicon PM-30 membrane, the dialysate was applied to a DEAE-11 column (100 ml) and the DPN-pyruvate adduct was purified as described above. Eighteen micromoles of material were recovered with a column yield of approximately 90%.

Proton magnetic resonance studies were carried out on a field-sweep Varian HR 220 spectrometer; signal to noise ratio was improved with a Nicolet 1074 computer. Samples were lyophilized twice from D₂O and run at a concentration of 0.01 M in Wilmad 100% D₂O at pH 8.1. Sample volumes were maintained at 0.25 ml using Wilmad Vortex plugs. Internal standards employed were tetramethylammonium chloride and trimethylsilyl sodium propionate (tetradecanoate). Ultraviolet spectra were obtained using a Perkin-Elmer Coleman 124 double beam spectrophotometer.
RESULTS AND DISCUSSION

In base, DPN+ and pyruvate form a reduced adduct according to the reaction scheme shown in Fig. 1; the structure of this adduct has been determined by Everse et al. (17), as well as Ozols and Marinetti (21). Also shown in Fig. 1 is the proposed structure of the abortive ternary complex of lactate dehydrogenase (e) which is a similar adduct but without subsequent ring closure.

If the abortive ternary complex of DPN+, enolpyruvate, and chicken heart lactate dehydrogenase (e) is a nicotinamide Cβ-substituted adduct as indicated in Fig. 1, it would be reasonable to assume that dissociation of this complex from the enzyme in base would yield a 340-nm-absorbing cyclized adduct (c) similar to that found in the chemical reaction, since such ring closures are catalyzed in base (22).

Fig. 2 shows that at pH 10.0 this sequence of reactions likely occurs. In this dual beam experiment, the initial rate of the enzymatic reaction was at least 10 times faster than the chemical reaction which was present in the reference cuvette. This experiment demonstrates that a binary compound (perhaps c) can be formed from the abortive ternary complex (e) in base.

In order to investigate the precise structure of the 340-nm-absorbing adduct produced by lactate dehydrogenase, we used high resolution proton magnetic resonance. Initially, we examined the structure of the DPN-cyanide adduct and the chemically produced DPN-pyruvate adduct (c) by proton magnetic resonance spectroscopy. Our studies of the cyanide adduct of DPN+ have revealed the existence of two forms of this adduct, one of the forms is present in a higher population than the other with a ratio of the forms of approximately 60:40 (23). We have also found by analogy to DPNH that the higher population species corresponds to the A side addition of cyanide to DPN+ and that the lower population form results from a B side addition. Consistent with this finding is the fact that dithionite reduces DPN+ preferentially on the A side of the nicotinamide ring (24).

On the basis of high and low populations of the diastereomeric forms of these DPN+ adducts in water, we assigned the proton magnetic resonance spectra of the A and B side addition products of DPN+ and pyruvate. The analysis of the chemically produced DPN-pyruvate adduct (c) has been simplified by the fact that only two major forms of this adduct exist. Since there are two asymmetric carbon atoms in this adduct, one at the pyridine Cβ position and one α to the carbonyl group, there are potentially four magnetically distinguishable forms. The fact that we observed only two forms reveals that the initial attack at the nicotinamide Cβ position (in b) predetermined the stereochemistry of the subsequent ring closure (in c).

Fig. 3 shows the PC6H region of the proton magnetic resonance spectrum for the chemically produced DPN-pyruvate adduct. The top of this figure shows computer simulations of the two forms of this adduct. It should be noted that the spectra of the chemically produced adduct results from the superposition of the spectra of Forms I and II except that Form I, the A side adduct, occurs in a higher population than Form II, the B side adduct. The ratios of Form I to Form II of the pyruvate adducts when formed at room temperature are very similar to those observed for the cyanide adduct and are approximately 60:40.

At the bottom of Fig. 3 is the same region of the proton magnetic resonance spectra but for the adduct produced by heart lactate dehydrogenase at pH 10.0. It is evident that the enzymatically produced adduct corresponds structurally to the chemically synthesized one except that only Form I is present, the A side addition product. Although it is not shown here, the remainder of the spectra of the enzymatic adduct is identical with that of Form I. The absence of Form II in the enzymatic reaction must be the result of enzymatic stereospecificity since this material was subjected to the same purification procedure as the chemically produced adduct. Furthermore, the DEAE-11 column purification gave approximately a 90% yield of the ap-

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8 Unpublished results.
but instead of a hydride transfer, the methylene group adds to pyruvate. However, a proton is probably also extracted. Dehydrogenase (25). With the normal substrate, lactate, histidine probably extracts a proton with a concomitant hydride addition also confirms the suggestion that the abortive ternary complex (c), which leads to this cyclized binary adduct (c), is a nicotinamide C4 substituted adduct. The nicotinamide C4 substitution has also been implied by x-ray studies (25). Furthermore, since the cyclized adduct produced by the heart lactate dehydrogenase is that resulting from an A side addition of pyruvate on the nicotinamide of DPN+ we feel it is reasonable to conclude that the ternary complex (e) likewise is an A side addition adduct. Based on the conclusion that the end form of pyruvate adds to the A side of the nicotinamide ring, it seems reasonable to propose a mechanism by which heart lactate dehydrogenase catalyzes abortive ternary complex formation. This information also confirms the suggestion that the abortive ternary complex (e), which leads to this cyclized binary adduct (c), is a nicotinamide C4 substituted adduct. The nicotinamide C4 substitution has also been implied by x-ray studies (25).

The fact that the heart lactate dehydrogenase does catalyze the formation of the cyclized DPN-pyruvate adduct (c) as shown by these proton magnetic resonance studies indicates that the reaction sequence given in Fig. 2 actually occurs. This information also confirms the suggestion that the abortive ternary complex (e), which leads to this cyclized binary adduct (c), is a nicotinamide C4 substituted adduct. The nicotinamide C4 substitution has also been implied by x-ray studies (25). Furthermore, since the cyclized adduct produced by the heart lactate dehydrogenase is that resulting from an A side addition of pyruvate on the nicotinamide of DPN+ we feel it is reasonable to conclude that the ternary complex (e) likewise is an A side addition adduct.

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The abortive ternary complex consisting of DPN+, enolpyruvate, and lactate dehydrogenase (e) closely resembles the transition state intermediate in the normal hydride transfer reaction.

REFERENCES

9. TIEKHAAR, R., and MEANG, J. E. (1973) Biochemistry 12, 2067

3 One should imagine the structure of the formed abortive ternary complex of Fig. 4 and the transition state of the normal substrate reaction.

4 Since the reduced uncyclized pyruvate adducts of DPN+ are unsaturated in solution, it is impossible to determine Kp or K values for these compounds using standard techniques. Kp values can be obtained, however, if the binding and dissociation constants can be determined. For an approximation of the binding rate constant we used the value of 8 X 10^-6 M-s^-1 as determined for the binding rate of (AcPy)DPNH to chicken heart lactate dehydrogenase (26). The dissociation rate constant of the reduced uncyclized pyruvate adduct of DPN+ from the abortive ternary complex can be roughly determined by the recovery of the enzymatic activity in the presence of high concentrations of DPNH after abortive ternary complex formation. Calculated from stop-flow measurements (14) this value is less than 0.1 s^-1. Thus Kp < 0.1 s^-1 / 8 X 10^-6 M-s^-1 < 10^-8 M.
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