Mechanistic Probes for Enzymatic Reactions. Oxidation–Reduction Indicators as Oxidants of Intermediary Carbanions (Studies with Aldolase, Aspartate Aminotransferase, Pyruvate Decarboxylase, and 6-Phosphogluconate Dehydrogenase)†

Michael J. Healy and Philipp Christen*

ABSTRACT: A number of carbanionic enzyme–substrate intermediates were found to exhibit pronounced susceptibility toward oxidation. Thus, enzyme–substrate intermediates of fructose-1,6-diphosphate aldolase from rabbit muscle, of aspartate aminotransferase from pig heart, of 6-phosphogluconate dehydrogenase from yeast, and of pyruvate decarboxylase from yeast are all oxidized by suitable redox indicators. Substantial reduction of these indicators occurs only in the presence of both enzyme and substrate. The oxidation of the aldolase–substrate intermediate was examined in detail employing the oxidation–reduction indicators phenazine methosulfate, 2,6-dichlorophenolindophenol, hexacyanoferrate(III), porphyrindin, and porphyrexide. The initial rate of reduction of the indicators depends on the concentration of substrate and follows apparent Michaelis–Menten kinetics. Analogous to the reaction of tetranitromethane reported previously (Christen, P., and Riordan, J. F. (1968), *Biochemistry 7*, 1531) the carbanion–enamine intermediate of dihydroxyacetone phosphate with aldolase ($^-CHOHC{=}N^+HRCH_2OPO_3^{2-}$; H_2NR = lysyl residue of aldolase) is thought to be the reactive species. The product of oxidation of this intermediate with hexacyanoferrate(III) is hydroxypyruvaldehyde phosphate ($CHOCOCH_2OPO_3^{2-}$). Electron acceptors thus may be employed as mechanistic probes to detect carbanionic intermediates in enzymatic reactions. Moreover, the ready susceptibility of carbanionic intermediates to oxidation by various *in vitro* oxidants raises the possibility that similar "dehydrogenase" actions of these enzymes might occur *in vivo* with natural electron acceptors.

Intermediates of enzymatic reactions can be demonstrated by means of specific reactivities which certain groups of the enzyme–substrate complex acquire transiently in the course of catalysis (Christen, 1970). Thus, the carbanion–enamine intermediate suggested by hydrogen isotope exchange studies (Rose and Rieder, 1955; Bloom and Topper, 1956; Rutter and Ling, 1958) to occur in the mechanism of action of fructose-1,6-diphosphate aldolase (*cf.* Rose and Rose, 1969; Horecker, 1970) proved to be selectively reactive toward tetranitromethane (Christen and Riordan, 1968; Riordan and Christen, 1969). Recent examination of this reaction has shown that tetranitromethane oxidizes the carbanion intermediate of dihydroxyacetone phosphate formed with aldolase ($^-CHOHC{=}NH^+RCH_2OPO_3^{2-}$; H_2NR = lysyl residue of aldolase) to hydroxypyruvaldehyde phosphate ($CHOCOCH_2OPO_3^{2-}$) with concomitant production of nitrite and nitroformate (Healy and Christen, 1972b). In the present study this susceptibility of the carbanionic aldolase–substrate intermediate to oxidation is expressed further utilizing oxidation–reduction indicators as oxidants. Several redox indicators are reduced to their leuco forms by the aldolase dihydroxyacetone phosphate intermediate. Similar oxidizable enzyme–substrate intermediates have been found with other enzymes whose mechanisms are thought to involve carbanion intermediates, indicating the general applicability of suitable electron acceptors to mechanistic studies of enzyme reactions (Healy and Christen, 1972a).

Experimental Section

Materials

Fructose-1,6-diphosphate aldolase (E.C. 4.1.2.13) from rabbit muscle, L-glycerol-3-phosphate dehydrogenase (E.C. 1.1.1.8), triosephosphate isomerase, glyoxalase I (*S*-lactoylglutathione methylglyoxallyase, E.C. 4.4.1.5), and 6-phosphogluconate dehydrogenase from yeast (E.C. 1.1.1.44), pyruvate decarboxylase from brewer's yeast (E.C. 4.1.1.1), fructose 1,6-diphosphate tetracyclohexylammonium salt, NADH, and NADPH were obtained from Boehringer; carboxypeptidase A (treated with diisopropyl fluorophosphate) and D-ribulose 5-phosphate disodium salt were obtained from Sigma; reduced glutathione, tetranitromethane, 2,6-dichlorophenolindophenol, brilliant Cresyl Blue, porphyrindin, porphyrexide, ferricytochrome *c*, and sodium pyruvate were obtained from Fluka; potassium hexacyanoferrate(III) and Methylene Blue were obtained from Merck; phenazine methosulfate and DL-*erythro*-β-hydroxyaspartic acid were obtained from Calbiochem; AG1-X4-Cl anion exchange resin, 200–400 mesh, was obtained from Bio-Rad. The α subform of cytoplasmic aspartate aminotransferase was isolated from pig heart by a modification of the method of Banks *et al.* (1968) (Zaoralek and Christen, 1972[1]). Dihydroxyacetone phosphate (the dicyclohexylammonium salt of the dimethyl ketal) was chemically synthesized and converted into dihydroxyacetone phosphate as reported by Ballou and Fischer (1956).

† From the Biochemisches Institut der Universität Zürich, CH-8032 Zürich, Switzerland. *Received July 3, 1972.* This work was supported by Schweizerischer Nationalfonds, Grant No. 3.680.71.

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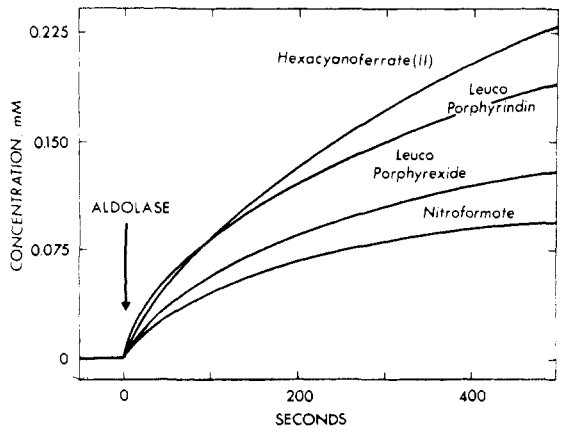


FIGURE 1: Reduction of redox indicators and of tetranitromethane in the presence of dihydroxyacetone phosphate and aldolase. The reactions were followed by recording the change of absorbance difference between sample and reference cuvets. Both cuvets contained 1.43 mM oxidized indicator or tetranitromethane, respectively, in 0.1 M Tris-Cl (pH 8.0); in addition, the sample cuvette contained 1.1 mM dihydroxyacetone phosphate. At zero time, aldolase (final concentration 0.055 mg/ml = 0.35 μM in a total volume of 1.4 ml) was added to both sample and reference cuvets. The concentrations of leuco indicators formed in the course of the reaction were calculated from absorbance readings at the wavelength corresponding to λ_{max} of the indicator employed using the values of molar absorptivities given in Table I. The indicator can be readily reoxidized; addition of hydrogen peroxide to a reaction mixture of aldolase, substrate, and hexacyanoferrate(III) that has been decolorized completely restores the initial concentration of hexacyanoferrate(III). For the reaction of tetranitromethane which produces nitroformate, absorbance at 350 nm was recorded; ϵ_{350} of nitroformate = 14,400 M^{-1} cm^{-1} (Glover and Landsman, 1964).

TABLE I: Oxidation–Reduction Indicators. Molar Absorptivities and Redox Potentials.

Indicator	λ_{max} (nm)	$\epsilon_{\lambda_{max}}$ (M^{-1} cm^{-1})	E_0' (V)[a]
Porphyrexide	420	670[b] (1440)[c]	0.725
Porphyrindin	585	1,210[b] (990)[c]	0.565
Hexacyanoferrate(III)	420	1,000[b] (1000)[c]	0.360
Ferrocytochrome *c*	550	27,700[b,d]	0.255
2,6-Dichlorophenol-indophenol	610	20,800[b,e]	0.217
Phenazine methosulfate[f]	387[a]	23,800[a,b]	0.080
Brilliant Cresyl Blue	632[a,b]		0.047
Methylene Blue	688[a,b]		0.011

[a] Loach (1968); E_0' represents the electrode potential of the half-oxidized–half-reduced dye at pH 7.0. [b] pH 8.0. [c] pH 6.0. [d] Margoliash and Schejter, 1966. [e] Armstrong (1964). [f] Reduction followed by coupling to cytochrome *c* (see legend of Table III).

Methods

Enzyme activities and concentrations were measured spectrophotometrically; the specific activities of aldolase (measured according to Bergmeyer *et al.*, 1970a), aspartate aminotransferase (Karmen, 1955), 6-phosphogluconate dehydrogenase (Bergmeyer *et al.*, 1970b), and pyruvate decarboxylase (Bergmeyer *et al.*, 1970c) were 10, 322, 11, and 20 units/mg (at 25°), respectively. For the digestion of aldolase by carboxypeptidase A (Drechsler *et al.*, 1959) the crystal suspension of aldolase (1 ml; 10 mg/ml) in ammonium sulfate was centrifuged and the sediment was dissolved in 1 ml of 0.1 M Tris-Cl–0.5 M NaCl (pH 7.6). After addition of 0.5 mg of carboxypeptidase A the solution was left at 4° for 2 hr and then dialyzed overnight against 0.05 M Tris-Cl (pH 8.0); the specific activity toward fructose 1,6-diphosphate fell to 1 unit/mg. Aldolase concentration was determined from the absorbance at 280 nm, $A_{1\,cm}^{1\,mg/ml}$ = 0.91 (Baranowski and Niederland, 1949). The molecular weight of aldolase is 158,000 (Kawahara and Tanford, 1966). Dihydroxyacetone phosphate was determined with glycerolphosphate dehydrogenase and NADH (Bücher and Hohorst, 1970). Hydroxypyruvaldehyde phosphate was isolated from the final reaction mixture (see Table II) by passing an aliquot (1 ml) onto an AG1-X4 chloride exchange column (8.4 × 0.6 cm) equilibrated with distilled water. The column was eluted with 4 ml of water and then with 0.2 M HCl. The first fraction (volume 1.5 ml) at the acid front was analyzed for hydroxypyruvaldehyde phosphate with glyoxalase I using reduced glutathione as cosubstrate (Weaver and Lardy, 1961). Tetranitromethane was diluted in ethanol before use to give a 33.6 mM solution. The molar absorptivities of the redox indicators (Table I) were determined from fresh weighed-in solutions in 0.2 M Tris-Cl (pH 8.0) and in 0.2 M sodium citrate (pH 6.0). Porphyrindin was standardized through reduction with ascorbic acid (Chinard and Hellerman, 1954). Kinetic measurements and visible–ultraviolet absorption spectra were obtained at 25° with a Unicam SP-1800 recording spectrophotometer. Measurements under nitrogen were carried out with a tonometer using a Cary Model 15 spectrophotometer. The rates of reduction of the indicators and of nitroformate production reported in this study are initial rates and were obtained from the slope of the tangent to the absorbance *vs.* time curve 6–10 sec after the enzyme had been added to the reaction mixture. The rates were corrected for the reduction of indicators by the enzyme in the absence of substrate and likewise by the substrate in the absence of enzyme.

Results

Addition of catalytic amounts of aldolase to a mixture of dihydroxyacetone phosphate and any one of the oxidation reduction indicators hexacyanoferrate(III), porphyrindin, or porphyrexide instantaneously initiates progressive reduction of the indicator to its leuco form (Figure 1). The reduction of the indicator requires the presence of both dihydroxyacetone phosphate and aldolase; in a mixture of indicator with either substrate or enzyme alone the indicator color remains virtually unchanged. The indicators are also reduced when fructose 1,6-diphosphate replaces dihydroxyacetone phosphate as substrate. Thus, the reactions of the redox indicators proceed quite similarly to the reaction of tetranitromethane reported previously (Christen and Riordan, 1968), where nitroformate is produced and which is shown for comparison (Figure 1).

Quantitative analysis shows that concomitant with indicator reduction equivalent amounts of substrate are oxidized. Thus, for each mole of hexacyanoferrate(III) reduced exactly 0.5 mol of dihydroxyacetone phosphate is consumed (Table II). The resulting product of oxidation was identified as hydroxypyruvaldehyde phosphate ($CHOCOCH_2OPO_3^{2-}$) and was isolated from the reaction mixture by anion exchange chro-

TABLE II: Oxidation of Dihydroxyacetone Phosphate to Hydroxypyruvaldehyde Phosphate in the Presence of Aldolase and Hexacyanoferrate(III).[a]

Compound	μmol
Hexacyanoferrate(III) reduced	54.0
Dihydroxyacetone phosphate consumed	27.4
Hydroxypyruvaldehyde phosphate isolated[b]	23.8

[a] Since hexacyanoferrate(III) is an inhibitor of aldolase (Birkenhäger, 1960) aliquots (20 μl) of 0.1 M potassium hexacyanoferrate(III) were added successively at intervals of about 3 min to a cuvet containing 27.4 μmol of dihydroxyacetone phosphate and 1 mg of aldolase in 0.15 M Tris-Cl (pH 8.0) with a total volume of 2.6 ml. The same amount of aldolase was added every 15 min (final volume 3.3 ml). The reaction (followed by the decrease in absorbance at 420 nm, see Table I) abruptly stopped after a total amount of 54 μmol of hexacyanoferrate(III) had been reduced. For isolation and determination of the product hydroxypyruvaldehyde phosphate see the section on Methods. [b] As demonstrated in a study of the analogous reaction of tetranitromethane a secondary product, 5-ketofructose 1,6-diphosphate, is formed through reversible aldolase-catalyzed condensation of dihydroxyacetone phosphate and hydroxypyruvaldehyde phosphate (Healy and Christen, 1972b). We assume that this product is also formed during the reaction with hexacyanoferrate(III). However, the reaction with hexacyanoferrate(III) was carried out to complete depletion of dihydroxyacetone phosphate; any 5-ketofructose 1,6-diphosphate was eventually cleaved again by aldolase and dihydroxyacetone phosphate was irreversibly oxidized.

matography with a yield of 87% of the initial dihydroxyacetone phosphate (Table II).[2]

The kinetic characteristics of the reduction of the indicators are consistent with their interaction with an enzyme-bound intermediate of the substrate. The initial rate of formation of leuco indicator (see Methods) is proportional to the concentration of enzyme and follows saturation kinetics with respect to dihydroxyacetone phosphate concentration (Figure 2a). Double reciprocal plots of the initial rates of reduction of the redox indicators *vs.* the concentration of dihydroxyacetone phosphate yield straight lines (Figure 2b). The substrate concentrations resulting in half-maximal rates of indicator reduction (K_m') as determined from the double reciprocal plots vary only by a factor of 2 when either porphyrindin, hexacyanoferrate(III), or tetranitromethane is employed as indicator (Table III). With carboxypeptidase A treated aldolase the rates of oxidation of fructose 1,6-diphosphate and of dihydroxyacetone phosphate by hexacyanoferrate(III) are about the same as those measured employing the native enzyme (Table III), though the rate of aldol cleavage is reduced about 20-fold by digestion with carboxypeptidase (Drechsler *et al.*, 1959). The ability to oxidize aldolase-bound substrate seems restricted to electron acceptors with relatively high potential

[2] The enzyme is serving as a catalyst and is not involved in the stoichiometry of the reaction. The relatively slow decrease of the reaction rate with time (Figure 1) as reflected by the curvature of the progression curve is probably due to inactivation of the enzyme protein independent of the indicator reduction, *e.g.*, through modification of sulfhydryl groups (*cf.* Riordan and Christen, 1968).

TABLE III: Kinetic Constants of the Aldolase-Catalyzed Oxidation of Dihydroxyacetone Phosphate by Redox Indicators.

Redox Indicator	Substrate	Aldolase	k'_{cat} (min^{-1})[a]	K_m' (mM)[b]
Porphydrindin[c]	DHAP[d]	Native	390	0.022
Hexacyanoferrate(III)[c]	DHAP	Native	94	0.015
Hexacyanoferrate(III)[c]	DHAP	CP-ALD[d]	94	
Hexacyanoferrate(III)[e]	$FruP_2$	Native	69	
Hexacyanoferrate(III)[e]	$FruP_2$	CP-ALD	75	
Porphyrexide[f]	DHAP	Native	70	
Tetranitromethane[g]	DHAP	Native	71	0.036
2,6-Dichlorophenol-indophenol[h]	DHAP	CP-ALD	0.25	
Phenazine methosulfate (coupled to cytochrome *c*)[i]	DHAP	Native	0.23	
Phenazine methosulfate (coupled to cytochrome *c*)[i]	DHAP	CP-ALD	0.25	

[a] k'_{cat} is the molecular activity of aldolase for oxidation of dihydroxyacetone phosphate. All indicators are two-electron acceptors, except hexacyanoferrate(III), porphyrexide, and cytochrome *c*. Thus, per mole of dihydroxyacetone phosphate oxidized, 2 mol of these indicators is reduced (see Table III). [b] K_m' is the substrate concentration at which the initial rate of indicator reduction is half-maximal. [c] Conditions as in Figures 1 and 2. The values for k'_{cat} and K_m' were obtained from Figure 2. [d] Abbreviations used are: DHAP, dihydroxyacetone phosphate; $FruP_2$, fructose 1,6-diphosphate; CP-ALD, aldolase pretreated with carboxypeptidase A (see Methods). [e] 0.67 mM hexacyanoferrate(III), 2.0 mM $FruP_2$ (K_m = 0.061 mM; Rutter *et al.*, 1963), 0.1 M Tris-Cl (pH 8.0), 0.03 mg/ml of aldolase (total volume 3.0 ml). [f] Conditions as in Figure 1. [g] 0.43 mM tetranitromethane; data from Christen and Riordan (1968). [h] 0.33 mM 2,6-dichlorophenolindophenol, 0.8 mM DHAP, 0.1 M Tris-Cl (pH 8.0), 0.25 mg/ml of enzyme (total volume 3.0 ml). [i] 0.082 mM ferricytochrome *c*, 0.82 mM phenazine methosulfate, 1.92 mM DHAP, 0.16 M Tris-Cl (pH 8.0), 0.3 mg/ml of enzyme (total volume 1.22 ml). The reduction was followed at 550 nm, the peak of the α band of reduced cytochrome *c* (*cf.* Table I). Without phenazine methosulfate no reduction of cytochrome *c* occurred.

(*cf.* Tables I and III). Thus, the two indicators with the lowest redox potential, brilliant Cresyl Blue and Methylene Blue, gave no observable reaction with the aldolase–dihydroxyacetone phosphate intermediate, when examined in a tonometer under nitrogen. Cytochrome *c*, notwithstanding its relatively high redox potential, was also not reduced.

The enzyme-catalyzed oxidation of substrate by redox indicators is not limited to aldolase but is noticed also with several other enzymes thought to form carbanionic enzyme–substrate intermediates analogous to aldolase. Thus, addition of aspartate aminotransferase (α subform of cytoplasmic enzyme from pig heart) to a solution of the pseudosubstrate DL-*erythro*-β-hydroxyaspartate and hexacyanoferrate(III) leads to a progressive reduction of the indicator (Figure 3). No reaction takes place with the substrate before the addition of the enzyme and, conversely, no reaction is observed when the enzyme is added to the indicator in the absence of the substrate.

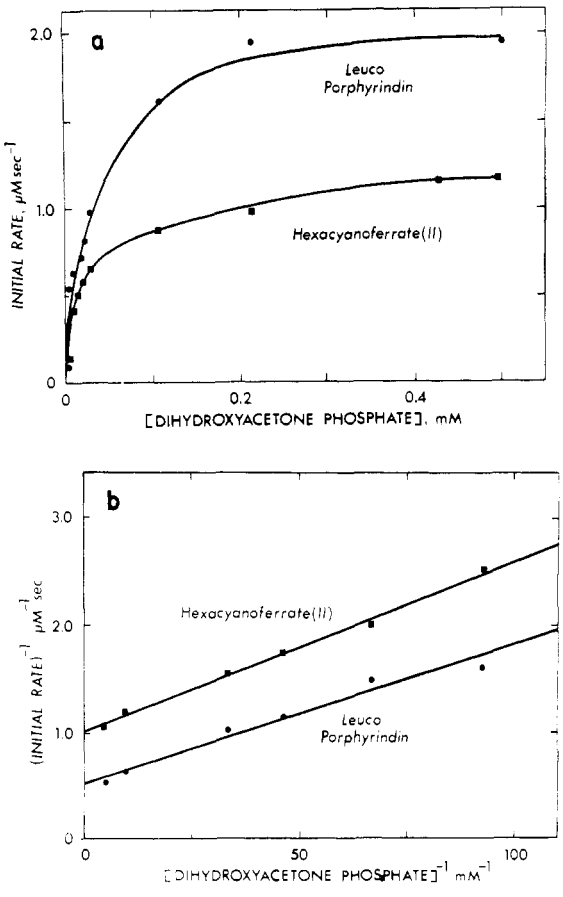


FIGURE 2: (a) Rates of reduction of porphyrindin and hexacyanoferrate(III) by aldolase at varying dihydroxyacetone phosphate concentrations. Conditions, except for substrate concentrations, were as in Figure 1. Initial rates were determined as described under Methods. (b) Double reciprocal plots of the rates of reduction of porphyrindin and hexacyanoferrate(III) *vs.* concentration of dihydroxyacetone phosphate. Data from Figure 2a.

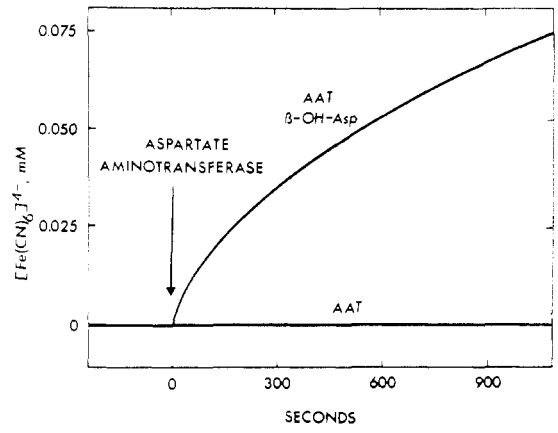


FIGURE 3: Reduction of hexacyanoferrate(III) in the presence of aspartate aminotransferase and DL-*erythro*-β-hydroxyaspartate. (1) AAT, β-OH-Asp. At zero time aspartate aminotransferase (AAT) (final concentration, 0.35 mg/ml) was added to a cuvet containing potassium hexacyanoferrate(III) (0.72 mM), DL-*erythro*-β-hydroxyaspartic acid (β-OH-Asp) (2.1 mM) in Tris-Cl (0.1 M, pH 8.0) in a final volume of 1.39 ml. The formation of hexacyanoferrate(II) was calculated from the decrease in absorbance at 420 nm (Table I). (2) AAT. When enzyme is added to a solution of the indicator without β-OH-Asp virtually no reaction occurs. In (1) and (2) the reference cuvets contained hexacyanoferrate(III) in the same concentration as the sample cuvets.

Similarly, when 6-phosphogluconate dehydrogenase from yeast (Figure 4) is added to a mixture of tetranitromethane and ribulose 5-phosphate more nitroformate is produced than there is formed in the reference cuvet containing tetranitromethane and ribulose 5-phosphate but no enzyme (curve ENZ, R5P). This slight, but significantly enhanced production of nitroformate may be attributed to an interaction of the enzyme and the substrate since in the presence of the enzyme alone (curve ENZ) significantly less nitroformate is produced. The effect of the enzyme–substrate intermediate on nitroformate production is markedly amplified when, in addition to enzyme and substrate, NADPH is also present (curve ENZ, NADPH, R5P). The effect is noticeable over and above the nitroformate production which is due to direct reaction of tetranitromethane with NADPH (curve ENZ, NADPH).

An analogous reaction is shown by pyruvate decarboxylase from yeast (Figure 5). Porphyrindin, porphyrexide, and tetranitromethane are readily reduced in the presence of pyruvate and pyruvate decarboxylase but not when either pyruvate, acetaldehyde, or the enzyme alone is present.

Discussion

The four enzymes, fructose-1,6-diphosphate aldolase, aspartate aminotransferase, 6-phosphogluconate dehydrogenase, and pyruvate decarboxylase, employed in this study catalyze rather diverse reactions but are related to one another by their mechanisms of action which involve the formation of a carbanionic intermediate consequent to the cleavage of a C–C or C–H bond. Examination of nonenzymatic systems has indicated that carbanions can be readily oxidized (*cf.* Russell and Bemis, 1966; Jagur-Grodzinski and Szwarc, 1969; Guthrie, 1969; Rynd and Gibian, 1970); it is this general property of carbanions that provides the basis for chemical probing (Christen, 1970) of such intermediates in enzyme mechanisms. As illustrated by the examples discussed below carbanionic intermediates of enzymatic reactions have—at least in the present cases—a sufficient lifetime and are adequately nucleophilic to be trapped by suitable electron acceptors.

Aldolase. The reduction of oxidation–reduction indicators to their leuco forms in the presence of aldolase and dihydroxyacetone phosphate (Figure 1) is analogous to the reaction of tetranitromethane with the carbanionic enzyme–substrate intermediate of aldolase reported previously (Christen and Riordan, 1968; Riordan and Christen, 1969; Healy and Christen, 1972b). The interaction of the redox indicators with an enzyme substrate intermediate can be inferred from the observations that (1) the reaction occurs only when aldolase *and* substrate are present in the reaction mixture, (2) the rate of indicator reduction is proportional to the concentration of the enzyme, and (3) the rate follows saturation kinetics with respect to substrate concentration (Figure 2).[3]

The reaction mechanism of aldolase is presumed to proceed as in eq 1 (*cf.* Rose and Rose, 1969; Horecker, 1970) (H_2NR = lysyl residue of aldolase). Since the reduction of the redox indicators by the aldolase–substrate intermediate occurs with both fructose 1,6-diphosphate and dihydroxyacetone

[3] The difference between K_m' (~0.025 mM, Table III) and K_m (2.1 mM, Rutter *et al.*, 1963) for dihydroxyacetone phosphate may be attributed to the different conditions employed. The K_m for the condensation reaction was determined in the presence of glyceraldehyde 3-phosphate which was absent in the reaction with the redox indicators.

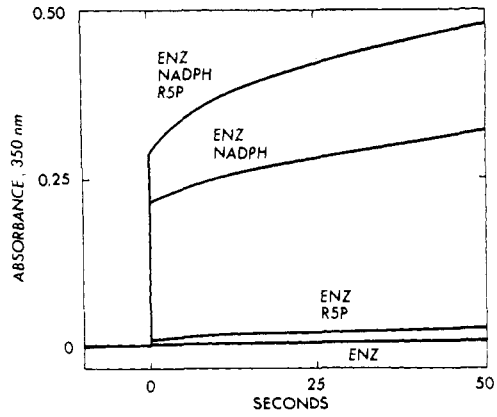


FIGURE 4: Reaction of the ternary system of 6-phosphogluconate dehydrogenase-D-ribulose 5-phosphate-NADPH with tetranitromethane. Both sample and reference cuvets contained 0.34 mM tetranitromethane in imidazole chloride (50 mM, pH 7.0) (λ_{350} of nitroformate 14,400 M^{-1} cm^{-1} (Glover and Landsman, 1964)). In addition, the following components were present in a final volume of 1.0 ml. (1) ENZ, NADPH, R5P. 6-Phosphogluconate dehydrogenase (ENZ, final concentration 4 μg/ml) and NADPH (final concentration 0.05 mM) were added at zero time to the sample cuvet containing D-ribulose 5-phosphate (R5P, final concentration, 0.34 mM). Absorbance was recorded against a reference cuvet containing R5P in buffer. (2) ENZ, NADPH. Ribulose 5-phosphate absent from sample and reference cuvets, otherwise same additions as in (1). (3) ENZ. R5P. No NADPH added, otherwise as in (1). (4) ENZ. Enzyme added to sample cuvet.

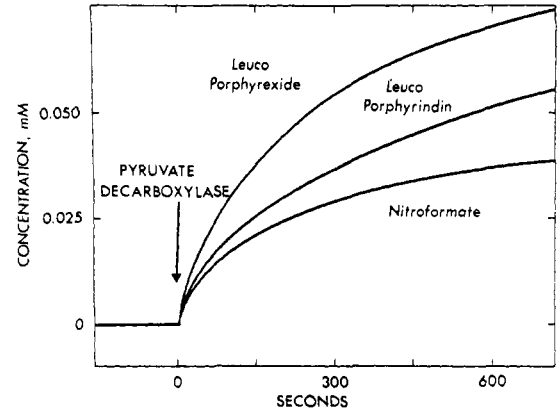
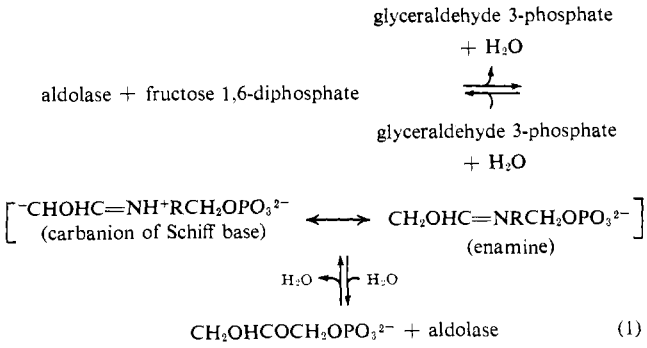


FIGURE 5: Reduction of electron acceptors in the presence of pyruvate decarboxylase and pyruvate. At zero time pyruvate decarboxylase (final concentration 15 μg/ml) was added to both sample and reference cuvets containing oxidized indicator or tetranitromethane (0.77 mM) in sodium citrate (0.1 M, pH 6.0, final volume 1.3 ml), the sample cuvet containing sodium pyruvate (7.7 mM) in addition. The difference in absorbance between the cuvets was followed as a function of time and transformed into concentration of leuco indicator (*cf.* legend of Figure 1). At pH 6.0 acetaldehyde did not react significantly with the indicators or with tetranitromethane.



(1)

phosphate, the postulated reductive intermediate must be located in a segment of the reaction sequence common to both substrates, *i.e.*, between the carbanion of dihydroxyacetone phosphate and the dissociation of dihydroxyacetone phosphate from the enzyme. Carboxypeptidase A digestion of aldolase decreases the rate of protonation of the dihydroxyacetone phosphate carbanion about 20-fold so that this step becomes rate limiting in the cleavage of fructose 1,6-diphosphate (Rose *et al.*, 1965). In contrast, the rate of reduction of hexacyanoferrate(III) in the presence of fructose 1,6-diphosphate is slightly increased for carboxypeptidase-treated aldolase as compared to the native enzyme (Table III), indicating that the carbanion, the intermediate occurring prior to the protonation step, is indeed the reductive species.[4]

The formation of hydroxypyruvaldehyde phosphate as the oxidation product is consistent with this scheme. The same compound has also been identified as the product of the reaction of tetranitromethane with the carbanionic intermediate (Healy and Christen, 1972b). The stoichiometry of the reaction where 2 mol of hexacyanoferrate(III) is reduced per mol of dihydroxyacetone phosphate oxidized (Table II) indicates the absence of any significant side reactions. The oxidation of the aldolase intermediate is very specific with respect to the substrate (Christen and Riordan, 1968) but rather nonspecific with regard to the nature of the oxidant. A series of redox indicators with widely varying structures can participate in the reaction, though with different rates (Table III). A certain minimal value of the redox potential of the oxidant ($E_0^{pH\ 7} \simeq 0.08$ V, see Tables I and III) appears to be a prerequisite for the oxidation of the aldolase-substrate intermediate. However, steric properties probably contribute to determining the rate of oxidation, *e.g.*, the unreactivity of cytochrome *c* may be caused by steric hindrance.

Aspartate Aminotransferase. A carbanionic intermediate in the reaction of aspartate aminotransferase has been postulated previously on the basis of data obtained from nonenzymatic model systems (Bruice and Topping, 1963) and from spectroscopic evidence (Jenkins, 1964). In analogy to studies with a nonenzymatic pyridoxal-glutamate system (Christen and Riordan, 1968) a tetranitromethane-reactive intermediate could be demonstrated using the substrate analog *erythro*-β-hydroxyaspartate (Shlyapnikov and Karpeisky, 1969). The present finding of a reduction of hexacyanoferrate(III) by an aminotransferase-β-hydroxyaspartate intermediate (Figure 3) demonstrates that this presumably carbanionic intermediate (Jenkins, 1964) is not only reactive toward tetranitromethane, but, rather similar to the aldolase intermediate, represents a generally oxidizable species.

6-Phosphogluconate Dehydrogenase. Studies of the stereospecificity of the 6-phosphogluconate dehydrogenase reaction including the stereospecific hydrogen isotope exchange of ribulose 5-phosphate catalyzed by this enzyme suggested the occurrence of an enol intermediate of ribulose 5-phosphate (Lienhard and Rose, 1964). The reaction of tetranitromethane

[4] The accelerating effect of carboxypeptidase A treatment of aldolase on the rate of oxidation of dihydroxyacetone phosphate is considerably more pronounced (220% of the initial value) when tetranitromethane is employed as an oxidant (Christen and Riordan, 1968). A possible reason for this difference may relate to the inhibitory effect of hexacyanoferrate(III) on the fructose 1,6-diphosphate cleavage activity of aldolase (Birkenhäger, 1960).

with an enzyme–ribulose 5-phosphate complex (Figure 4) supports the hypothesis of the existence of a distinct enol intermediate. The identity of the enol and the tetranitromethane-reactive intermediates is suggested by the fact that both hydrogen exchange (Lienhard and Rose, 1964) and substantial reaction with tetranitromethane require the presence of NADPH (Figure 4). This analogy supports the hypothesis of Lienhard and Rose (1964) that NADPH does not participate in its reducing capacity in the hydrogen exchange reaction in the absence of CO_2 but rather is an integral part of the structure of the active site that catalyzes the enolization of ribulose 5-phosphate.

Pyruvate Decarboxylase. 2,6-Dichlorophenolindophenol (Holzer and Goedde, 1957) and phenazine methosulfate (Holzer and Crawford, 1960) have been reported to serve as electron acceptors in the oxidation of pyruvate to acetate catalyzed by pyruvate decarboxylase from yeast. The present finding of additional analogous reactions with tetranitromethane, porphyrindin, and porphyrexide (Figure 5) demonstrates the general applicability of oxidants to trap the "activated acetaldehyde" intermediate which may be formulated as a carbanion (Breslow, 1958).

Conclusions

There exist, besides the oxidoreductases, a number of enzymes that form oxidizable substrate intermediates, and thus are capable of catalyzing oxidation reactions in the presence of suitable electron acceptors. The catalytic efficiency of aldolase as a "dehydrogenase" is quite comparable with its effect as a C–C lyase; under the present conditions the molecular activity for oxidation of dihydroxyacetone phosphate is about 5% of that for the cleavage of fructose 1,6-diphosphate (Table III). Nevertheless, there are important differences between the enzymes demonstrated here to be capable of catalyzing oxidation reactions and the true dehydrogenases. There is no indication that in the case of aldolase and the other enzymes studied here the electron acceptors would saturate the enzyme, *i.e.*, would bind appreciably to the enzyme–substrate complex. Furthermore, only oxidants with a relatively high redox potential ($E_0^{pH\ 7} > 0.08$ V, Tables I and III) are capable of serving as electron acceptors. However, these differences might be of a rather quantitative nature and the first steps of the mechanism of action of the present enzymes and that of certain oxidoreductases might be quite similar. Such mechanistic relationships have also been emphasized on the basis of other lines of evidence that indicate the prominent role of proton transfer and therewith of general acid and base catalysis in both redox and nonredox enzyme reactions (*cf.* Hamilton, 1971). In agreement with this view for D-amino acid oxidase, evidence has recently been presented indicating that the α hydrogen of the substrate is removed as a proton (Walsh *et al.*, 1971).

Many other oxidizing agents besides the ones employed in this study may prove to be suitable probes for intermediary carbanions.[5] Electron acceptors might be applied as mechanistic probes both to the detection of carbanionic intermediates and, by employing redox indicators differing in, *e.g.*, size, structure, or redox potential, to the exploration of more detailed features of such intermediates and their active-site environments. Furthermore, the ready susceptibility of carbanionic enzyme–substrate intermediates to oxidation by *in vitro* oxidants raises the possibility that similar oxidation reactions catalyzed by these enzymes might occur *in vivo* with natural electron acceptors.

[5] The reaction of certain oxidizing agents, *e.g.*, hexacyanoferrate(III) or tetranitromethane (*cf.* Healy and Christen, 1972b), might also be quantified by determining the concomitant release of protons.

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Abortive Complexes of α-Amylases with Lanthanides†

Alexander Levitzki* and Jacques Reuben

ABSTRACT: Apoamylases from both hog pancreas and *Bacillus subtilis* were prepared by removing the tightly bound Ca^{2+}. In both cases lanthanide ions fail to activate the enzyme. Furthermore, in both cases the rare earth ions do not compete for the essential Ca^{2+} binding site and therefore do not inhibit the Ca^{2+} activation process. *B. subtilis* α-amylase possesses two Ca^{2+} binding sites, one of which can bind Gd^{3+}, with an additional site for the lanthanide ion.

The lanthanide series constitutes a group of 14 elements in which the electronic f shell is progressively filled providing them with diverse spectral and magnetic properties. Because of the close similarity in ionic radii of the lanthanides with the calcium ion it has been suggested (Birnbaum *et al.*, 1970; Darnall and Birnbaum, 1970; Williams, 1970) that these ions can be used as replacements for calcium and used as probes for the calcium binding sites in biological systems. This possibility seems to be highly attractive since the calcium ion has filled electron shells and thus is devoid of spectral and magnetic properties which can be used to probe its mode of interaction with its binding site. The lanthanides seem to open a new dimension in the study of numerous biological systems which require calcium for their function and stability. Indeed it has been recently reported that some lanthanide ions can replace Ca^{2+} in a few systems such as the enzyme α-amylase from *Bacillus subtilis* (Smolka *et al.*, 1971), thermolysin from *B. thermoproteolyticus* (Colman *et al.*, 1972), and as substitutes for Ca^{2+} in the activation of trypsinogen to trypsin (Darnall and Birnbaum, 1970).

α-Amylases are known to be calcium metalloenzymes in which the metal ion is absolutely required for functional integrity, yet the specific role of the metal has not been delineated. We wish to report in this article our attempts to substitute the calcium ion by lanthanide ions in α-amylases from hog pancreas and *B. subtilis*.

Materials and Methods

Soluble starch Analar was obtained from British Drug House. Charcoal (Darco G60) was obtained from the British Drug House and treated according to Whistler and Durso (1950). Solutions of lanthanide were prepared by dissolving the metal sesquioxides (Alfa Inorganics) in HCl. All other chemicals used were of the highest purity available.

B. subtilis α-amylase was purchased from Calbiochem. The enzyme was dialyzed against Hepes[1] (0.02 M, pH 6.9) in the cold and centrifuged (12,000*g*) to remove insoluble material. The enzyme was found to be over 95% homogeneous using gel electrofocusing (Wrigley, 1968).

Hog pancreatic α-amylase was prepared according to Loyter and Schramm (1962) except for the charcoal–calcite stage. The α-limit dextrins were removed from the enzyme by charcoal treatment in batches at pH 8.5 using washed Darco G60 charcoal activated by heating at 100° overnight, before use. The activity of α-amylases was measured at pH 6.9 as described previously (Loyter and Schramm, 1962). The specific activity of the enzyme was found to be 1550 units/mg using the modified Bernfeld assay (Loyter and Schramm, 1962).

Protein concentration was measured using the extinction coefficients $A^{280}_{1\%} = 24.3$ for pancreatic α-amylase and $A^{280}_{1\%} = 25.3$ for the *B. subtilis* α-amylase (Stein *et al.*, 1964).

The preparation of apo-α-amylases was performed by continuous dialysis against 0.01 M EDTA (pH 7.0 at 4°) using a

† From the Departments of Biophysics (A. L.) and Isotope Research (J. R.), The Weizmann Institute of Science, Rehovot, Israel. *Received August 8, 1972.*

[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mops, 2-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DFP, diisopropyl phosphorofluoridate.