[CONTRIBUTION FROM THE CONVERSE MEMORIAL LABORATORIES, HARVARD UNIVERSITY]

The Oxygen Exchange Reaction Catalyzed by the Succinic-thiokinase[1]

BY LOWELL P. HAGER

RECEIVED APRIL 10, 1957

Succinic-thiokinase has been purified from extracts of aerobically grown *Escherichia coli*. The purified enzyme catalyzes a rapid exchange of oxygen between inorganic phosphate and succinic acid in the presence of adenosine triphosphate and Coenzyme A. A comparison of the rate of the oxygen exchange with the rate of the inorganic phosphate–adenosine triphosphate exchange indicates the oxygen exchange reaction proceeds independently of the over-all reaction. The relationship between the oxygen exchange reaction and the nature of the intermediate in this reaction is discussed.

Introduction

The substrate phosphorylation reaction accompanying the oxidation of α-ketoglutaric acid has been shown by Kaufman,[2] *et al.*, and Hift,[3] *et al.*, to occur in the nucleotide disphosphate dependent conversion of succinyl CoA[4] to succinate according to the reaction

$$\text{succinyl CoA} + \text{XDP} + \text{P} \rightleftharpoons \text{succinate} + \text{XTP} + \text{CoA} \quad (1)$$

The enzyme catalyzing this reaction has been termed the succinate phosphorylating enzyme (P enzyme) and more recently Beinert,[5] *et al.*, have proposed the name succinic-thiokinase for this enzyme. The XDP and XTP in equation 1 may be either guanosine or inosine di- and triphosphates with the purified heart muscle enzyme[6] or adenosine di- and triphosphate with the enzyme purified from spinach.[7]

Kaufman[8] has studied the mechanism of the phosphorylation reaction through a series of exchange reactions and proposed an enzyme bound S-phosphoryl CoA as the intermediate in equation 1. Free succinyl phosphate was eliminated as an intermediate by the fact that the purified enzyme would catalyze neither ATP nor succinyl CoA formation from synthetic succinyl monophosphate.[8,9] The formation of an enzyme-bound succinyl phosphate was not eliminated by the exchange data but the inability to trap labeled succinate in carrier succinyl phosphate when carrying out the over-all reaction indicated that no dissociation occurred if such an enzyme product were formed.

In contrast to these experiments, Cohn[10] has reported the isolation of succinate containing oxygen[18] formed during the oxidation of ketoglutarate by rat liver mitochondria in the presence of O^{18} phosphate. This result is consistent with the intermediary formation of succinyl phosphate. The reason for the observed inhibition of the oxygen transfer by dinitrophenol, however, is not apparent since this substrate level phosphorylation reaction is insensitive to this reagent.[11]

A subsequent paper will report the purification of and exchange data with succinic-thiokinase from *Escherichia coli*. The purified enzyme catalyzed a rapid oxygen exchange between inorganic phosphate and succinate in the presence of ATP and CoA.

Experimental

Methods and Materials.—Disodium ATP was purchased from the Sigma Chemical Company. CoA was obtained from Pabst Laboratories. Oxygen[18] phosphate was prepared by the method of Cohn[12] with O^{18} water purchased from Dajac Laboratories. Phosphorus[32] phosphate was obtained from the Biophysics Laboratory of Harvard Medical School.

Oxygen 18:16 ratios were determined on CO_2 samples in the Consolidated Nier Mass Spectrometer. Phosphate and succinate oxygen were converted to CO_2 employing the Unterzaucher method for oxygen analysis as developed by Doering and Dorfman.[13]

A gas flow counter was used for radioactivity determinations. The extent of orthophosphate incorporation into ATP was determined after isobutyl alcohol extraction of inorganic phosphate as ammonium phosphomolybdate[14] and measurement of the radioactivity in the organic bound phosphate.[15]

Succinate was isolated from the reaction mixtures by continuous ether extraction after acidification to *p*H 1.0 with H_2SO_4. The ether was removed by evaporation on a steam cone and the succinic acid dissolved in a minimum amount of warm water. The solution was placed on a 10-gram Dowex-1-hydroxide column and the column washed with 100 ml. of water and 100 ml. of 0.01 *M* formic acid. The succinic acid was then eluted with 0.1 *M* formic acid and the samples lyophilized to remove the formate. The succinic acid isolated in this manner was completely free of inorganic and organic phosphate and assayed 90 ± 10% pure in the succinic oxidase assay.[16]

The enzyme was assayed by the hydroxylamine trapping method.[11] The enzyme units of activity are expressed as micromoles of succinohydroxamic acid formed per 30 minutes. Specific activity is expressed as units per milligram of protein. Protein was determined by the optical method of Warburg and Christian.[17]

Enzyme Purification.—Crude extracts obtained from aerobically grown *E. coli*, Crookes strain, proved to be an excellent source of succinate phosphorylating enzyme. The specific activity of crude extracts of heart muscle, spinach and *E. coli*, as measured by hydroxamic acid are recorded in Table I. Assuming the turnover number of the enzyme

(1) This work was supported in part by a grant from the William F. Milton Fund of Harvard University and by the American Cancer Society.

(2) S. Kaufman, C. Gilvarg, O. Cori and S. Ochoa, *J. Biol. Chem.*, **203**, 869 (1953).

(3) H. Hift, L. Ouellet, J. W. Littlefield and D. R. Sanadi, *ibid.*, **204**, 565 (1953).

(4) Abbreviations employed: CoA, Coenzyme A; ATP, adenosine triphosphate; ADP, adenosine diphosphate; P, inorganic phosphate.

(5) H. Beinert, D. E. Green, P. Hele, O. Hoffman-Ostenhof, F. Lynen, S. Ochoa, G. Popjak and R. Ruyssen, *Science*, **124**, 614 (1956).

(6) D. R. Sanadi, D. M. Gibson, P. Ayengar and M. Jacob, *J. Biol. Chem.*, **218**, 505 (1956).

(7) S. Kaufman and S. G. A. Alivisatos, *ibid.*, **216**, 141 (1955).

(8) S. Kaufman, *ibid.*, **216**, 153 (1955).

(9) S. Kaufman, *Arch. Biochem. & Biophys.*, **50**, 506 (1954).

(10) M. Cohn, "Phosphorus Metabolism," Vol. I, Johns Hopkins Press, Baltimore, Md., 1951, p. 374.

(11) S. Kaufman, "Methods in Enzymology," Vol. I, Academic Press, New York, N. Y., 1955, p. 719.

(12) M. Cohn and G. R. Drysdale, *J. Biol. Chem.*, **216**, 831 (1955).

(13) W. von E. Doering and E. Dorfman, THIS JOURNAL, **75**, 5595 (1953).

(14) I. Berenblum and E. Chain, *Biochem. J.*, **32**, 295 (1938).

(15) M. Grunberg-Manago, P. J. Ortiz and S. Ochoa, *Biochim. Biophys. Acta*, **20**, 269 (1956).

(16) W. W. Umbreit, R. H. Burris and J. F. Stauffer, "Manometric Techniques and Tissue Metabolism," Burgess Publishing Co., Minneapolis, Minn., 1949, p. 166.

(17) O. Warburg and W. Christian, *Biochem. Z.*, **310**, 384 (1941).

to be the same regardless of the source, the *E. coli* extract contains approximately 150 times more enzyme than the heart extract and 20 times more enzyme than the spinach extract.

TABLE I

COMPARISON OF SOURCES FOR SUCCINATE PHOSPHORYLATION ENZYME

Source of enzyme	Specific activity of initial crude extract, μmole hydroxamic acid/30 min./mg. protein
1. Heart muscle	0.03[a]
2. Spinach	0.22[b]
3. *Escherichia coli*	4.1

[a] Ref. 2. [b] Ref. 7.

The growth conditions for the microörganism are the same as those used to obtain active pyruvate and α-ketoglutarate dehydrogenase systems.[18] The cells were harvested by centrifugation on the Sharples centrifuge and stored in the deep freeze as a cell paste. Extracts of the frozen cell paste were prepared by suspending 10 g. of frozen cell paste in 15 ml. of *M*/50 *p*H 7.4 phosphate buffer (hereafter referred to as phosphate buffer) and subjecting the mixture to sonic oscillation in a Raytheon 9KC oscillator for 40 minutes. Cell debris was removed by centrifugation at 0° for 1 hour at top speed in a Servall SS1 angle head centrifuge. Several supernatants obtained in this manner were pooled and stored in the deep freeze for subsequent purification. All fractionation steps were carried out at 0° and the intermediate fractions were stored in the deep freeze with practically no loss of activity.

First Ammonium Sulfate Fractionation.—The crude extracts were brought to 0.25 ammonium sulfate saturation by the addition of 14.6 g. of ammonium sulfate per 100 ml. of extract. The precipitated protein was removed by centrifugation (top speed of SS1 Servall for 15 minutes) and discarded. The supernatant was brought to 0.75 ammonium sulfate saturation by the addition of 34.3 g. of ammonium sulfate per 100 ml. of original extract. The resulting precipitate was centrifuged in the Servall and suspended in phosphate buffer $^5/_6$ the volume of the original extract.

Protamine Precipitation.—Nucleic acid and inactive protein was removed from 0.25–0.75 saturated ammonium sulfate fraction by the addition of protamine sulfate. The ammonium sulfate fraction was adjusted to *p*H 5.9 by the slow addition of 1 *N* acetic acid. Twenty ml. of 2% *p*H 5.0 protamine sulfate per 100 ml. of enzyme solution were added slowly with vigorous stirring. The inactive precipitate was removed by centrifugation and discarded.

Second Ammonium Sulfate Fractionation.—The supernatant from the protamine treatment was brought to 0.38 ammonium sulfate saturation by the addition of 26.6 g. of ammonium sulfate per 100 ml. of supernatant. The precipitate was removed by centrifugation and discarded. The supernatant was brought to 0.52 ammonium sulfate saturation by the addition of 8.8 g. of ammonium sulfate per 100 ml. The precipitate was removed by centrifugation and suspended in phosphate buffer in $^1/_6$ the volume of the original extract.

Third Ammonium Sulfate Fractionation.—The 0.38–0.52 saturated ammonium sulfate fraction was brought to 0.5 saturation by the addition of an equal volume of saturated ammonium sulfate. The precipitate was removed by centrifugation and discarded. The supernatant was brought to 0.55 saturation by the addition of 11 ml. of saturated ammonium sulfate per 100 ml. of supernatant. The precipitate was removed by centrifugation and suspended in phosphate buffer in 0.4 volume of the previous fraction. The fraction contained 22% of the original activity and was approximately ten-fold purified over the initial extract. A summary of the purification on a representative lot is given in Table II.

The purified enzyme is completely dependent upon ATP, CoA and succinic acid for synthesis of succinohydroxamic acid in the presence of 0.5 *M* hydroxylamine. Additionally, the purified enzyme catalyzed the arsenolysis of succinyl CoA,[6] as measured by the arsenate dependent liberation of CoA sulfhydryl groups but did not catalyze the arsenolysis of synthetic succinyl phosphate.

(18) S. Korkes, "Methods in Enzymology," Vol. I, Academic Press, New York, N. Y., 1955, p. 719.

TABLE II

PURIFICATION OF *E. coli* ENZYME

Fraction	Volume, ml.	Protein, mg.	Units	Specific activity	% Units
Crude extract	620	17,500	72,000	4.1	100
First ammonium sulfate	500	12,000	62,000	5.2	89
Protamine supernatant	587	6,390	56,000	10.0	78
Second ammonium sulfate	100	1,690	44,700	24.0	62
Third ammonium sulfate	40	388	15,800	40.6	22

Exchange Results

The incubation of the purified enzyme with O^{18}-labeled phosphate in the presence of succinate, ATP, CoA led to a rapid exchange of oxygen between succinate and phosphate (Table III). The phosphate was additionally labeled with phosphorus^{32} to indicate the extent of over-all reversal of reaction 1. The use of relatively large amounts of enzyme in this experiment to achieve 50% equilibration between inorganic phosphate and ATP revealed a slow P-ATP exchange in the absence of succinate (line 5, Table III). This probably is due to the presence of an unknown contaminating enzyme since the succinic-thiokinase enzyme isolated from other sources has an absolute succinate requirement for the phosphate-ATP exchange. Within experimental error, the O^{18} exchange reaction between inorganic phosphate and succinate is completely dependent upon ATP, CoA and enzyme. A comparison of the rate of O^{18} exchange into succinate and the P^{32} exchange into ATP indicates that the oxygen exchange reaction can occur independently of the over-all reaction 1. Assuming that one succinate oxygen would be replaced during complete reversal of reaction 1, the theoretical per cent. O^{18} exchange into succinate would be $^1/_4$ the value of the P^{32} exchange into ATP (100% exchange representing replacement of all four succinate oxygen atoms). In contrast to this prediction, the O^{18} exchange into succinate is some fivefold greater than the theoretical value (Table III). This finding implies the reversible formation and cleavage of an intermediate compound, independent of the over-all reversal of reaction 1.

Discussion

The failure to detect or accumulate an intermediate in the succinate activation reaction has necessitated the study of exchange reactions as an indirect approach to an elucidation of the mechanism of this reaction. On the basis of the substrate and cofactor requirements for the succinate–succinyl CoA exchange, ADP–ATP exchange and P–ATP exchange, Kaufman has suggested the formation of an enzyme bound S-phosphoryl CoA intermediate.[8] More direct evidence for the biological occurrence of an S-phosphoryl CoA derivative is offered by the experiments of Wolleman and Feuer,[19] who report the isolation of an enzyme from brain tissue which catalyzes the transfer of the terminal

(19) M. Wolleman and G. Y. Feuer, *Acta Physiol. Acad. Sci. Hung.*, **7**, 343 (1955).

TABLE III

P^{32} AND O^{18} PHOSPHATE EXCHANGE WITH ATP AND SUCCINATE

The complete system contained 100 μM Tris buffer, *p*H 7.4, 10 μM $MgCl_2$, 10 μM cysteine, 5 μM ATP, 50 μM K succinate, 1 μM CoA, 5 μM K phosphate (73,400 c.p.m./μM P^{32} and 5.05% excess O^{18}) and 1.6 mg. enzyme. Incubated at 30° for 1 hour.

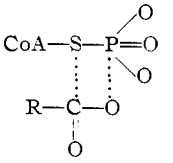
	Additions	$P^{32}O_4$ ATP exchange C.p.m./μM	% exch.	PO_4^{18} succinate exchange 46/44 ratio of CO_2	Atoms % excess	%[a] exchange
1.	Complete system	20,650	56	0.00762	0.338	66.9
				.00744		
2.	Complete system –enzyme	0	0	.00422	.0	0
				.00421		
3.	Complete system –CoA	2,000	5	.00439	.024	4.7
				.00441		
4.	Complete system –ATP	0	0	.00441	.024	4.7
				.00447		
5.	Complete system –succinate	5,900	16	.00433	.010	2.0
				.00425[b]		

[a] Calculated on the basis of 100% exchange being equal to replacement of 4 oxygens of succinate by O^{18} from phosphate and correcting for the tenfold excess of succinate over phosphate. [b] Succinate added after acidification and extracted.

phosphate of ATP to CoA forming the S-phosphoryl compound according to equation

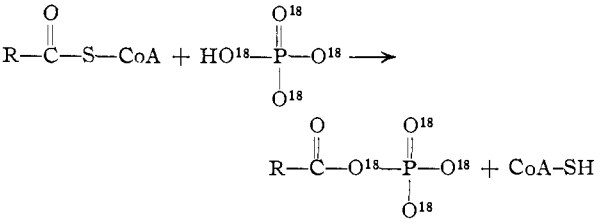
$$ATP + CoA\text{–}SH \longrightarrow ADP + COA\text{–}S\text{–}P$$

The oxygen exchange reaction shown to occur between phosphate and succinate defines specific requirements with respect to the postulated intermediary formation of S-phosphoryl CoA. Precisely, the O^{18}-exchange indicates that an oxygen atom from phosphate would have to be incorporated into the succinate without the possibility for exchange with water or other oxygen-containing species in the medium. Schematically, this can be formulated in bare essentials as a four center double displacement reaction.



The detailed mechanism of course could be much more complicated involving perhaps enzyme bound intermediates. The end-result, however, would have to conform with directed oxygen transfer between succinate and the postulated S-phosphoryl CoA.

The intermediate formation of an "enzyme–succinyl phosphate complex" followed by cleavage of the P–O bond with the formation of succinate and ATP also would account for the observed oxygen exchange. This explanation, however, makes no provision for the observed faster rate of O^{18} exchange between phosphate and succinate when compared to the P^{32} exchange between phosphate and ATP. The reversible formation and cleavage of succinyl phosphate from succinyl CoA and inorganic phosphate according to the equation shown does not predict O^{18} exchange until subsequent rupture of the P–O bond with formation of ATP and succinic acid. This would predict equal exchange rates for both O^{18} and P^{32}. Therefore, the alternative formation of an enzyme bound succinyl phosphate would have to provide in addition an equilibration mechanism for the carboxyl oxygens of succinic acid which would allow the oxygen exchange reaction to proceed at a faster rate than over-all reversal of reaction 1. Theoretically, this could be accomplished by cleaving and reforming the succinyl phosphate P–O bond while the succinyl phosphate is still complexed with the enzyme. Under these conditions the carboxyl oxygens of succinate would become equivalent.



There would appear to be a close analogy between the succinate phosphorylation reaction and the glutamine synthetase reaction with respect to the nature of the activated intermediate. The oxygen transfer studies of Boyer, *et al.*,[20] and Kowalsky, *et al.*,[21] reveal a transfer of oxygen from the γ-carboxyl of glutamic acid to inorganic phosphate in the glutamine synthetase reaction and led to the suggestion of γ-glutamyl phosphate as an intermediate in the synthetase reaction. Attempts by Levintow and Meister to demonstrate glutamine synthesis from ammonia and glutamyl phosphate, however, have yielded negative results.[22]

The final answer to the nature of the intermediate in the succinate phosphorylation reaction would appear to require the use of substrate amounts of enzyme and must await further purification of the enzyme. The high enzyme content of crude extracts of aerobically grown *E. coli*, compared to the heart and spinach extracts, suggests the use of this organism as an excellent enzyme source for extensive purification work.

CAMBRIDGE, MASSACHUSETTS

(20) P. D. Boyer, O. J. Koeppe and W. W. Luchsinger, THIS JOURNAL, **78**, 356 (1956).

(21) A. Kowalsky, C. Wyttenbach, L. Langer and D. E. Koshland, Jr., *J. Biol. Chem.*, **219**, 719 (1956).

(22) L. Levintow and A. Meister, *Federation Proc.*, **15**, 299 (1956).