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Direct Oxygen Transfer in Enzymic Syntheses Coupled to Adenosine Triphosphate Degradation[1]

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In the enzymic synthesis of acetyl-coenzyme A, oxygen from acetate appears in the phosphate group of the adenylate formed. In the enzymic synthesis of glutamine, oxygen from glutamate appears in the inorganic phosphate formed. These and other observations support the hypothesis that, in enzymic synthesis coupled to adenosine triphosphate degradation, oxygen transfer occurs from the substrate to a moiety cleaved from the adenosine triphosphate.

In the enzymic formation of 3-phosphoglycerate and of succinate coupled with inorganic orthophosphate (P_i) uptake, oxygen from the P_i appears in the carboxyl of the acid formed.[3] In the synthesis of adenosine triphosphate (ATP) coupled to the degradation of citrulline, oxygen from inorganic phosphate appears in the carbon dioxide formed.[4] In the coenzyme A transferase reaction, oxygen from the carboxyl of succinate appears in the acetoacetate formed.[5] These findings, together with considerations of probable reaction mechanisms, led to the hypothesis that, in enzymic syntheses coupled to ATP degradation, oxygen transfer occurs from the substrate to a moiety cleaved from the ATP, and that the moiety from the ATP in which the oxygen appears depends upon the nature of the enzymic activation. This paper presents studies on the enzymic syntheses of acetylcoenzyme A and of glutamine which support this hypothesis.

The synthesis of acetyl-coenzyme A according to equation 1[6] and of glutamine according to equation 2[7] represent the two distinct types of ATP cleavage known to occur in coupled enzymic syntheses, namely, cleavage to form adenylic acid (AMP) and pyrophosphate or to form adenosine diphosphate (ADP) and P_i. Exchange studies with radioactive substrates in the absence of net reaction have suggested that the reactions involve formation of intermediates with adenylate[6b] or with orthophosphate,[8] respectively.

$$\text{ATP} + \text{acetate} + \text{Coenzyme A} = \text{AMP} + \text{Acetyl-coenzyme A} + \text{pyrophosphate} \quad (1)$$

$$\text{ATP} + \text{glutamate} + \text{NH}_3 - \text{ADP} + \text{glutamine} + \text{P}_i \quad (2)$$

In experiments on acetyl-coenzyme A synthesis, using an enzyme preparation from rabbit heart,[9] oxygen from acetate-O^{18} appeared in the phosphate group of the AMP formed, as shown by the results given in Table I. An atom % excess O^{18} of 0.24 would have been expected in the phosphate from AMP if each mole of adenylate formed enzymically contained one oxygen from the acetate-O^{18}. The appearance of some O^{18} in the pyrophosphate fraction likely resulted from the presence of adenylate kinase in the enzyme preparation used.

These results give strong evidence that in the coupled synthesis of acetyl-coenzyme A the initial reaction of the ATP involves a nucleophilic displacement by an oxygen of a second reactant on the phosphorus of the phosphate attached to the 5-position of the ribose, with liberation of free pyrophosphate. The simplest reaction sequence would involve acetate as the second reactant, with intermediate formation of an adenylacetate; this possibility is in harmony with the recent demonstration by Berg[10] that adenyl acetate is a probable intermediate in the catalysis. Subsequent reaction with coenzyme A to form acetyl-coenzyme A would be expected to leave with the adenylate an oxygen originally present in the acetate. However, the isotopic data do not rule out participation of other intermediates, such as an oxygen containing group on the enzyme, which in subsequent reactions gains an oxygen from acetate. The results are more difficult to reconcile with the suggestion[6b] of intermediate formation of an enzymyl-coenzyme A derivative.

In experiments on glutamine synthesis, with use of an enzyme preparation from peas,[7a] oxygen from the γ-carboxyl of the glutamate appeared in the P_i formed, as shown by the results given in Table II. Within experimental error, the amount of O^{18} found in the Pi corresponds to that expected for transfer of one oxygen from the glutamate carboxyl. Similar results have been obtained in independent experiments by Kowalsky and Koshland,[11] who further showed that no oxygen from the glutamate appeared in the ADP formed. In the glutamine synthesis the initial reaction very likely involves a nucleophilic displacement on the phosphorus of the terminal phosphate; as with the acetyl-coenzyme A synthesis the simplest explanation would be intermediate formation of a glutamyl phosphate. Although the evidence does not favor participation of glutamyl phosphate as an intermediate,[12,7a] this possibility should be given further consideration.

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TABLE I

OXYGEN TRANSFER FROM ACETATE TO ADENOSINE MONOPHOSPHATE ACCOMPANYING THE ENZYMIC SYNTHESIS OF ACETYL-COENZYME A

Atom % excess O^{18} of the acetate used	Atom % excess O^{18} of the phosphates formed	
	Adenosine monophosphate	Pyrophosphate
0.00	0.01	0.01
.95	.21	.03

TABLE II

OXYGEN TRANSFER FROM GLUTAMATE TO INORGANIC ORTHOPHOSPHATE ACCOMPANYING THE ENZYMIC SYNTHESIS OF GLUTAMINE

Atom % excess O^{18} of the glutamate used	Atom % excess O^{18} of the orthophosphate formed
0.00	0.01
.76	.18

Experimental

O^{18}-Labeled Compounds.—Acetate labeled with O^{18} was prepared by use of the exchange between the carboxyl oxygen and the oxygen of water that occurs in acidic solution at elevated temperatures.[13] One ml. of glacial acetic acid and 2 ml. of H_2O (1.27 atom % excess O^{18}) were mixed and heated at 110–113° for 18 hours. The solution was neutralized to *p*H 7.5 with KOH, evaporated to dryness on a steam-bath, and heated overnight at 110° to give anhydrous potassium acetate. The amount of O^{18} in the acetate was determined by heating duplicate 40-mg. samples dissolved in 0.20 ml. of 7.5 *N* H_2SO_4 in a sealed tube at 125–130° for 48 hours. The sample was frozen, volatile constituents removed under high vacuum, the acetic acid in the distillate neutralized with anhydrous $Ba(OH)_2$, a portion of the water collected under high vacuum, and the O^{18} content of a 50-μl. sample of water determined as described previously by the sulfite–bicarbonate equilibration procedure. The acetate contained 0.88 atom % excess O^{18}.

Glutamic acid labeled in the γ-carboxyl with O^{18} was prepared by heating a mixture of 1 g. of L-glutamic acid, 0.45 ml. of concentrated HCl and 2.0 ml. of H_2O^{18} (1.27 atom % excess O^{18}) in a sealed tube at 125–128° for 36 hours; under these conditions an equilibrium mixture of glutamic acid and γ-pyrrolidonecarboxylic acid would be expected to be present.[14] The solution was neutralized to a *p*H of about 3 with NaOH (5 g. NaOH per 10 ml. H_2O), the precipitate collected on a small Büchner funnel and washed thoroughly with 50 vol. % ethanol. The product was recrystallized from ethanol–water. For determination of the amount of O^{18} in the γ-carboxyl group, 8.4 mg. of the glutamic acid was heated with a low flame in an evacuated tube and the water liberated by formation of the γ-pyrrolidonecarboxylic acid[15] was equilibrated with carbon dioxide by exposure to a hot platinum wire as described previously.[3a] The glutamic acid contained 0.76 atom % excess O^{18} in the γ-carboxyl group.

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Oxygen Transfer in Acetyl-coenzyme A Formation.—The enzyme preparation was a supernatant solution from frozen and thawed rabbit heart mitochondria prepared as described by Von Korff.[9] For convenience in estimation of the amount of reaction and to drive the reaction, use was made of the coupled reactions by which reduced diphosphopyridine nucleotide appears in amount equivalent to the acetyl-coenzyme A formed during the reaction.[9] The reaction mixture contained in 10-ml. final volume and at an initial *p*H of 9.0, 30 μmoles of potassium ATP, 50 μmoles of $MgSO_4$, 1.7 mg. of coenzyme A (Pabst), 10 μmoles of glutathione, 30 μmoles of diphosphopyridine nucleotide, 100 μmoles of potassium malate, 100 μmoles of potassium acetate, 400 μmoles of KCl, 500 μmoles of trishydroxymethylaminomethane, 1 μmole of ethylenediaminetetraacetate and 0.5 ml. of enzyme solution. Incubation was for 90 minutes at 37°; the extent of reduced diphosphopyridine nucleotide formation was followed by measurement of the absorbancy at 340 mμ using a portion of the mixture in an 0.05 cm. light path. For the experiment reported in Table I, with use of acetate-O^{18} approximately 13 μmoles of coenzyme A was formed; in a similar control experiment with non-isotopic acetate approximately 14 μmoles of acetyl-coenzyme A was formed.

At the end of incubation, the reaction mixtures were deproteinized by addition of 0.5 ml. of 70% perchloric acid, chilling and centrifugation. To the supernatant solution 0.5 ml. of concentrated HCl was added and the solution heated 10 minutes at 100° to hydrolyze labile phosphate compounds. The inorganic phosphate fraction, which contained phosphate derived from the pyrophosphate formed by reaction 1, was isolated as $MgNH_4PO_4$. The stable phosphate fraction, which contained the phosphate derived from adenosine monophosphate formed by reaction 1, was treated with alkaline phosphatase and the inorganic phosphate formed isolated as $MgNH_4PO_4$. These isolations and subsequent O^{18} determinations were made essentially as described previously.[3a] Separate experiments similar to those described above confirmed the transfer of oxygen from acetate to the adenylate and not to the pyrophosphate.

Oxygen Transfer in Glutamine Synthesis.—The enzyme was prepared according to the method of Elliott.[7a] The preparation was carried only through the protamine treatment (Stage 3) since the enzyme at this stage showed almost no detectable ATPase activity and was suitable for these studies. The extent of reaction was determined by measuring the amount of P_i formed during the reaction. There was no detectable phosphate formation in the absence of glutamate. The reaction mixture contained 2.5 ml. of 0.8 *M* trishydroxymethylaminomethane at *p*H 7.8, 2.5 ml. of 0.1 *M* sodium glutamate, 2.5 ml. of 0.05 *M* ATP, 0.5 ml. of *M* cysteine, 0.5 ml. of *M* $MgSO_4$, 0.5 ml. of *M* NH_4Cl and 2 ml. of enzyme solution. The glutamate, ATP, cysteine and NH_4Cl were adjusted to about *p*H 7.8 before addition to the reaction mixture. The reaction was allowed to run for 50 minutes at 30°. At the end of the incubation, the reaction mixtures were deproteinized with 3.75 ml. of 12% trichloroacetic acid and centrifuged. Analysis of the supernatant solutions showed that in the two experiments using glutamate-O^{18} and in a control experiment (glutamate-O^{16}) from 27.6 to 27.8 μmoles of P_i was formed. Thirty μmoles of carrier phosphate was added to each and the phosphate was isolated as the $MgNH_4PO_4$. The isolations and O^{18} determinations were carried out as described previously.[3a]

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