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The Citrate Condensing Enzyme of Pigeon Breast Muscle and Moth Flight Muscle*

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Citrate condensing enzyme has been purified from pigeon breast muscle and from moth (*Cecropia* and *Cynthia*) flight muscle. The preparations were homogeneous proteins as judged by ultracentrifugal data and starch gel electrophoresis. The kinetic behavior of the two preparations was studied using a newly developed assay for citrate condensing enzyme.

The study of the activity-structure relationship of a number of proteins has been aided by the isolation from various sources, of pure proteins having apparently identical functions. Theorell, his colleagues, and others[1] have studied the structure of cytochrome *c* from several sources and have found similarities in the structure of the peptide surrounding the point of attachment of the heme residue. The amino acid sequences for the same hormone from a number of sources have been determined and compared. From such studies the structure of the active site of these hormones has been deduced[2,3]. More information is now available for some hormones because chemical variants can be synthesized and their activities compared.

Aldolase[4], malate dehydrogenase[5] and phosphorylase[6] are examples of enzymes which have been purified from a number of sources and whose behavior and structure have been rather extensively studied. Though the citrate condensing enzyme occurs in high concentrations in a number of biological materials it has been highly purified only from pig heart tissue[7]. In our recent studies on citrate condensing enzyme[8] we had cause to compare the enzyme from a number of sources. This paper presents the procedures for the purification of citrate condensing enzyme from pigeon breast muscle and moth (*Cecropia* and *Cynthia*) flight muscle. We will report, in addition, on the sedimentation behavior and kinetic behavior of these proteins and a new method for the assay of citrate condensing enzyme.

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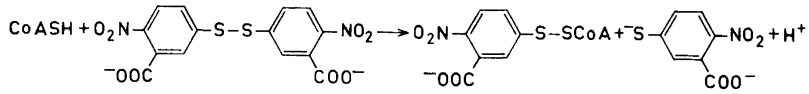
METHODS AND MATERIALS

The assay in which citrate condensing enzyme is coupled to malate dehydrogenase, as described by Ochoa[9], was used as the routine assay during purification. This assay measures the appearance of NADH at 340 mμ. In the assay of the moth muscle enzyme it was necessary to include 0.5 μmole of NaCN to inhibit a NADH oxidase that is present in early fractions. In addition to assays that have already been described[10], a new assay for citrate condensing enzyme has been developed. It is based on the reaction of CoASH with Ellman's[11] reagent 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). When a sulfhydryl compound such as CoASH reacts with this compound the following reaction occurs:



The mercaptide ion absorbs light at 412 mμ and has a molar absorbancy index of 13 600. Thus if acetyl CoA and oxalacetate react in the presence of citrate condensing enzyme and DTNB the rate of CoASH formation can be followed spectrophotometrically at 412 mμ. This assay has several advantages over earlier ones in that it is more sensitive, can be followed in a region where the other components do not absorb light and a wider concentration range of substrates can thus be tested in kinetic experiments on the enzyme. At low enzyme concentrations a slight lag period is observed (15 sec) in this reaction.

Our results (Table 1) show that under the conditions of the assay the rate of change of absorption at 412 mμ is proportional to enzyme concentration and

Table 1. Characteristics of the DTNB assay for citrate condensing enzyme. Except where that component is the variable, reaction mixtures were 0.2 M Tris-HCl pH 8.1, 1×10^{-4} M DTNB, 4.7×10^{-5} M acetyl CoA, 2.3×10^{-4} M oxalacetate and pig heart enzyme sufficient to give the indicated rates. Total volume was 1.0 ml in 1.0 cm light path cells. All rates are corrected to 25°C.

Condensing enzyme μg	ΔA/min 412 mμ
0.4	0.057
0.8	0.111
1.2	0.155
1.6	0.203
2.0	0.254

DTNB M × 10⁵	ΔA/min 412 mμ
1	0.061
2	0.119
3	0.153
5	0.178
8	0.177
10	0.178
200	0.178

Acetyl CoA determination μmoles/ml

μl Solution assayed	412 mμ	ml Solution assayed	233 mμ
20	4.5	0.1	5.7
30	4.7	0.2	5.3
50	4.7	0.4	4.9
100	4.7		

Oxalacetate determination μmoles/ml

412 mμ	Malate dehydrogenase 340 mμ
103	105

that change of DTNB concentration from 5×10^{-5} M to 2×10^{-3} M does not affect the rate of reaction. If the coupled enzyme assay of Ochoa *et al.*[9] is followed at 355 mμ (an isosbestic point for the DTNB change) no effect of the rate of the reaction is observed. If enzyme is titrated with DTNB or with other SH binding reagents, no effect on the rate of reaction is observed. Neither DTNB or 2-nitro-5-mercaptide benzoate reacts with acetyl CoA under the conditions of the assay. However, the initial velocities measured with the DTNB assay are from 10–30 % lower than assays which measure the disappearance of acetyl CoA and oxalacetate at 233 mμ when only the reaction components are present. The reaction rate at 233 mμ is non-linear for the whole reaction course while the DTNB assay gives a relatively long period of linear reaction rate. No explanation can be given at present for these discrepancies.

Kinetic measurements were performed using a Beckman DU monochromator, a Gilford optical density converter and a Minneapolis Honeywell recorder with a chart speed of 8 in/min. The cell compartments were thermostated and triplicate determinations were run at all concentrations. Acetyl CoA and oxalacetate concentrations were determined using the DTNB assay with limiting amounts of the component being assayed and an excess of other reaction components. Enzyme concentration was determined each day by assaying the preparation at high concentrations of acetyl CoA and oxalacetate. Temperature of the reaction mixture was determined at the end of each assay and corrections were applied assuming that the temperature dependence of the moth muscle and pigeon breast enzymes were the same as that for the pig heart enzyme[10]. Temperature corrections were routinely less than ± 10 %.

Sedimentation studies were carried out in a Spinco Model E centrifuge. Starch gel electrophoresis was performed as described earlier[12]. Protein was determined using the method of Warburg[13] or Lowry *et al.*[14]

Thoraces of the moths (*Cynthia* and *Cecropia*) were kindly supplied by Dr. David Shappirio, Department of Zoology, The University of Michigan. These were assumed to be essentially all flight muscle and no further disections were performed.

Purification of pigeon breast muscle citrate condensing enzyme. The method used for the purification of this enzyme was identical to the method we have described previously for the pig heart enzyme. The only difference in behavior that was observed was during the

Table 2. Purification of citrate condensing enzyme from pigeon breast muscle. The top half of the table represents a typical purification from 100 g of pigeon breast muscle. The bottom half of the table represents a purification where the elutes from three separate 100 g runs were combined and purified together. One unit of activity is one μmole of NADH formed per min in the coupled malate dehydrogenase assay. Specific activity are units per mg protein.

	Total activity (units)	Specific activity
KCl-EtOH Extract	2 900	0.08
50–70 % Ammonium sulfate	2 600	0.58
DEAE Eluate	1 540	–
Combined calcium phosphate eluate	4 750	8.9
70 % Ammonium sulfate ppt.	4 250	16.1
1st X's	2 420	30
Residue	900	10
2nd X's	1 750	50

Table 3. Purification of citrate condensing enzyme from moth *(Cynthia)* flight muscle. See Table 1 for units.

	Total Activity (units)	Specific Activity
H_2O Extract	4 200	0.83*
DEAE Eluate	3 500	2.1*
Calcium phosphate eluate	3 850	8.9
80 % Ammonium sulfate ppt.	2 580	15
Crystals		40†

† The crystals reported here represent the reprecipitation of a number of ammonium sulfate precipitates. A 30–50 % yield in the crystallization step is obtained.
* These extracts have materials which absorb strongly at 260 mμ so that Lowry's method is used for protein determination at these steps.

DEAE cellulose step. The pigeon breast enzyme is eluted with 0.008 M potassium phosphate buffer (pH 7.4), while the pig heart enzyme is not eluted until 0.018 M potassium phosphate buffer (pH 7.4). A summary of a typical purification is shown in Table 2.

Purification of moth flight muscle citrate condensing enzyme. Fifty grams of thoraces are homogenized for 2 min in 500 ml of ice cold water using a Waring blendor. The homogenate is centrifuged for 15 min at 20 000 g. The supernatant fluid is poured through cheese cloth. 200 gm (wet weight) of DEAE cellulose (equilibrated with 0.002 M potassium phosphate pH 7.4) are taken up in 500 ml H_2O and added to the extract. The purification of condensing enzyme then follows the procedure outlined for the pig heart enzyme except in the last ammonium sulfate step. Where the pig heart enzyme is recovered with 70 % ammonium sulfate saturation it is necessary to have 80 % ammonium sulfate saturation at pH 6.5 in order to precipitate all of the moth enzyme. A summary of the purification procedure is shown in Table 3.

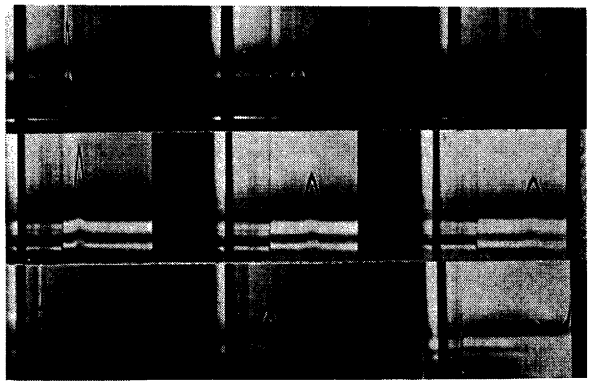


Fig. 1. Sedimentation of pig heart (top 2, 31 and 71 minutes), pigeon breast (middle 20, 52 and 68 minutes) and moth flight muscle (bottom 1, 17 and 76 minutes) citrate condensing enzymes. 59 780 RPM, 20°C, 0.05 M potassium phosphate pH 7.4.

RESULTS

Purity of the enzymes. Fig. 1 shows photographs of the sedimentation of the three citrate condensing enzymes. Only one symmetrical peak is seen in each case. The sedimentation coefficient ($s_{20,w}$) in Svedberg units, at infinite dilution was 4.5 for the moth enzyme, 6.2 for the pigeon enzyme and 6.1 for the pig enzyme. The final ammonium sulfate precipitation of these enzymes look "crystalline"; that is as the protein precipitates the solutions take on a characteristic silkiness or sheen that has been associated in the crystallization of other enzymes. We have examined these proteins by starch gel electrophoresis and when one-half of the gel is developed for protein staining material only a single band is visible in both cases. When the region of starch corresponding to the protein band is eluted from the other half of the starch gel, enzyme activity could be demonstrated. No difference was noted in the purification of the enzyme from the species *Cecropia* and *Cynthia.*

Kinetic studies. Figures 2 and 3 show Lineweaver-Burk plots for acetyl CoA at several oxalacetate concentrations for each enzyme. The apparent K_m of acetyl CoA in the case of the pigeon breast enzyme is 1.8×10^{-4} M and that for acetyl CoA for the moth flight muscle is 2.0×10^{-4} M. Insufficient data is available at present for an estimate of the apparent K_m's of oxalacetate. However, they seem to be of the magnitude of 10^{-6} M for the pigeon breast enzyme and 10^{-5} M for the moth muscle enzyme. The nature of the kinetics with the pigeon breast enzyme indicates that K_m and the association constant (K_s) for acetyl CoA may be equal[15], whereas this it not the case with the moth enzyme or the pig heart enzyme.

The kinetic behaviour of the pig heart enzyme was also determined using the new assay described here. The slopes of lines for $1/V_0$ *vs* 1/acetyl CoA at several

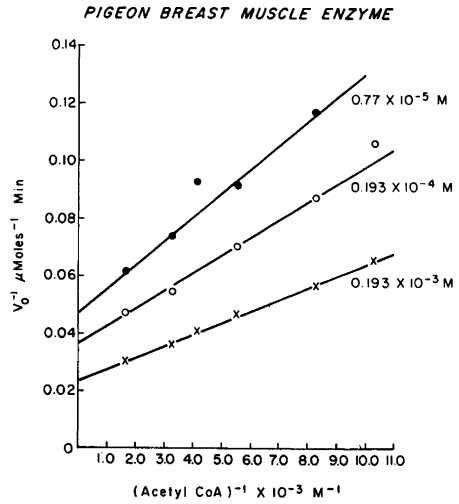


Fig. 2. Reaction mixtures contained acetyl CoA and oxalacetate at the concentrations noted and 0.2 M Tris-HCl pH 8.1 and 1×10^{-4} M DTNB. The reaction was initiated by the addition of enzyme and all velocities are reported on the basis of 0.34 mg of pure enzyme.

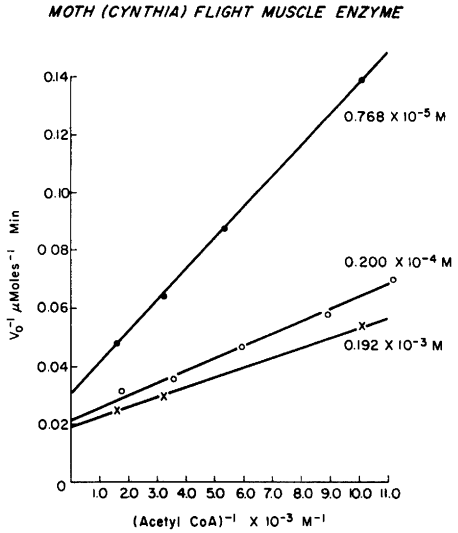


Fig. 3. See legend to Fig. 2.

oxalacetate concentrations were similar to our previous results in that they were almost equal. However, in the 233 mμ assay where the slopes decreased slightly with decreasing oxalacetate concentration, our results with the 412 mμ assay showed a very slight increase in slope with decreasing oxalacetate concentration. Since the 233 mμ assay is a less accurate one at low reaction rates we are inclined to place more reliance on our present results. In any case, the kinetic analysis for each of the three enzymes seems to be sufficiently different so that a common kinetic pathway seems unlikely.

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