IDENTIFICATION OF AN ESSENTIAL RESIDUE OF PIG HEART ACONITASE

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Summary: Treatment of aconitase with phenacyl bromide prior to activation with Fe(II) and reductant results in complete, irreversible enzyme inactivation. Inactivation is due to the alkylation of a cysteine residue at the active site of the enzyme, the inactivation being inhibited by the competitive inhibitor, tricarballylate. Active enzyme is similarly inactivated, citrate affording greater protection than tricarballylate.

Although citrate (isocitrate) hydrolyase [EC 4.2.1.3.] from pig heart, as well as from other sources, has long resisted purification to homogeneity (1), recent application of ion-exchange chromatography and isoelectric focusing has resulted in homogeneous** pig heart aconitase (2,3). The availability of pure pig heart aconitase makes possible investigation of structure-activity relationships, such investigations not being possible with inhomogeneous material. This report is then concerned with first identification of an essential cysteine residue of aconitase, this residue being S-alkylated by phenacyl bromide with concomitant loss of activity.

Materials and Methods

Aconitase was prepared and stored in 15 mM Tris Tricarballylate buffer at 2°C as described previously (2,3,5). At this point the enzyme contains 2Fe/mole and must be activated by the addition of ferrous ion and reductant, one g-atom Fe(II) being incorporated into the protein. The following experiments were performed on the activatable enzyme. The aconitase is then activated with a mixture of cysteine-ascorbate - ferrous ion and assayed at 26°C (2,3,5).

Phenacyl bromide was obtained from Aldrich Chemical Company and recrystallized from high boiling petroleum ether before use, mp. 50-51°C (uncorrected). α-[^{14}C] phenacyl bromide was synthesized on a millimole scale by the method of Cowper and Davidson (6) and Vogel (7). The product was recrystallized as above and stored in ether solution at -20°C. When used, aliquots were diluted with absolute ethanol and the concentration of bromide calculated from its extinction coefficient (9.26 mM^{-1} cm^{-1}) at 250 nm. The [^{14}C]-samples were counted by dissolving in 10 ml of INSTAGEL (Packard) and reading with a Nuclear Chicago Unilux II Scintillation Counter.

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**A previous preparation (4) has been shown (3) to be impure.

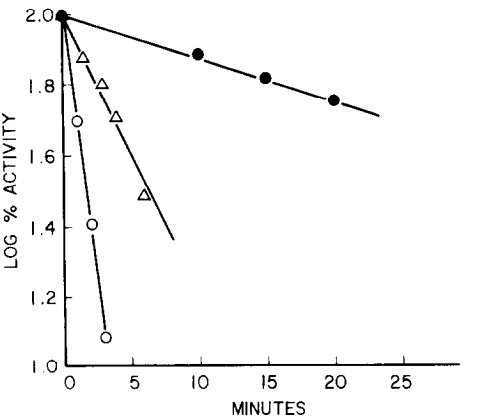

Figure 1. Kinetics of inactivation by phenacyl bromide at pH 7.8 and 2°C: 5.6μM aconitase, 0.6 mM phenacyl bromide , 40.5 mM Tris-acetate, and 20% ethanol in a final volume of one milliliter; tricarballylate-free (open circles), 40.5 mM Tris-acetate, pH 7.8; open triangles represent 0.01 mM and closed circles 0.045 mM Tris-tricarballylate; specified times 0.1 ml aliquots were added to 0.9 ml activating solution (5) and a 0.1 ml aliquot of this solution was assayed (2,3,5).

Tricarballylate-free enzyme was prepared by exhaustive dialysis at 2° against 45 mM Tris-acetate, pH 7.8, using an Amicon Model 10 Standard Stirred Cell and PM-10 membrane.

Reaction of α-[^{14}C] Phenacyl Bromide with Aconitase. A mixture of 0.0085 mM tricarballylate-free aconitase, 30mM Tris Acetate, 22% ethanol and 0.3 mM [^{14}C] phenacyl bromide were allowed to react for 30 minutes at pH 8.7 and 2°C at which time the enzyme could no longer be activated. (Another experiment performed contained 30 mM Tris tricarballylate instead of Tris acetate). Aliquots (0.5 ml) were treated with an equal volume of 20% trichloroacetic acid solution for 30 minutes at room temperature and precipitated protein was removed by membrane filtration (membrane filters, type GA-8, 25 mm diameter, Gelman Instrument Company). The membranes were washed with 5 x 15 ml portions of 10% trichloroacetic acid, 5 x 15 ml portions of absolute ethanol, and air-dried. The membranes were transferred to a scintillation vial, treated with 1 ml of hydroxide of Hyamine 10-X (Packard), 10 ml of INSTAGEL and counted.

Paper Chromatography of the Acid Hydrolysate of [^{14}C] Phenacyl Aconitase. Five mg (0.076 μmoles) of aconitase was phenacylated and worked up as above. Seven-tenths of a milligram of the [^{14}C] protein was removed from the membrane and hydrolyzed for 24 hours at 100°C (in vacuo) with 1 ml of 6N hydrochloric acid. The hydrolysate (0.35 mg) was spotted in Whatman 1MM paper and ascending chromatography run in methanol-water-pyridine (80:20:4) solvent mixture. For comparison purposes, α-[^{14}C] S-phenacyl-L-cysteine was prepared by the method of Glasel and coworkers (8) and also spotted from 6N HCl. For determination of radioactivity, 6 cm wide strips were cut into equal R_f zones and the segments were placed in a scintillation vial with 10 ml of INSTAGEL.

Preparation of [^{14}C] Phenacylated, Reduced and Carboxymethylated Aconitase and Its Tryptic Peptides. Tricarballylate-free aconitase (0.212 μmoles) was modified with [^{14}C] phenacyl bromide (1.05 μmoles, 1 x 10^6 cpm) in 3 ml of 45 mM Tris acetate, pH 8.7 and 1.0 ml ethanol at 2°C until the enzyme was no longer activa-

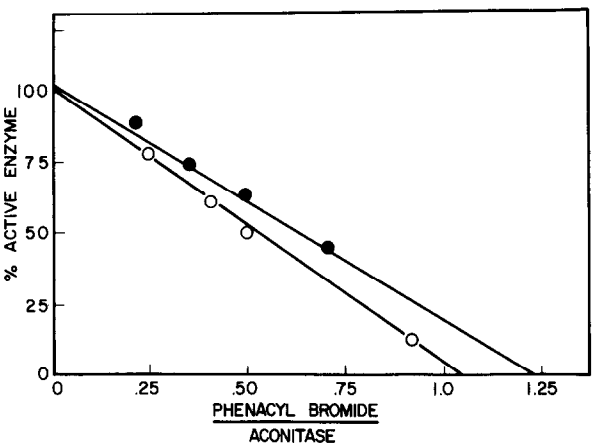

Figure 2. Reaction of limiting concentrations of phenacyl bromide with aconitase at 2°C: Reaction mixtures contained 11μM aconitase and 20% ethanol in a final volume of one milliliter; 12 mM Tris Tricarballylate, pH 9.8 (closed circles) and 22 mM Tris-acetate, pH 9.8 (open circles); reaction allowed to proceed to constant enzyme activity.

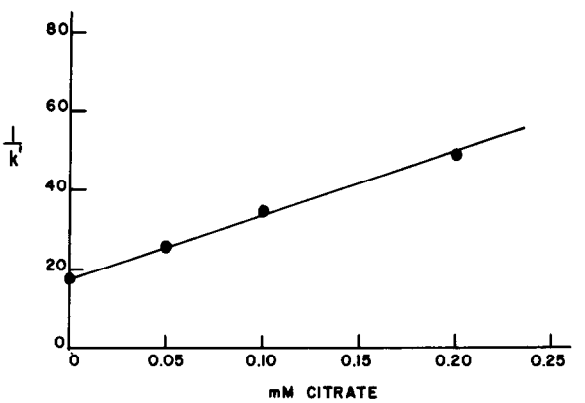

Figure 3. Effect of citrate on phenacylation rate: reciprocal first order slopes against citrate concentration. 46 μg active enzyme (5), 0.0092 μmoles phenacyl bromide, 20% alcohol, 67.5 mM Tris-acetate, pH 9.0, 2°, 1.5 mM Tris Tricarballylate, 1.0 ml total volume, 0.1 ml aliquots assayed directly.

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table, (30 minutes). The excess bromide was destroyed at addition of 1 ml of 60 mM cysteine, pH 8.5. After an additional 45 minutes at 2°C, the reagents were removed by dialysis against deionized water in an Amicon Standard Stirred Cell Model 52 using a PM-10 membrane. This was continued until the dialysate was free of radioactivity. The protein was lyopholized , reduced, and carboxymethylated as done previously (9) giving a final yield of 12 mg of protein. Trypsin hydrolysis of 4.3 mg of this protein was performed (9) using trypsin free of chymotrypsin (Calbiochem).

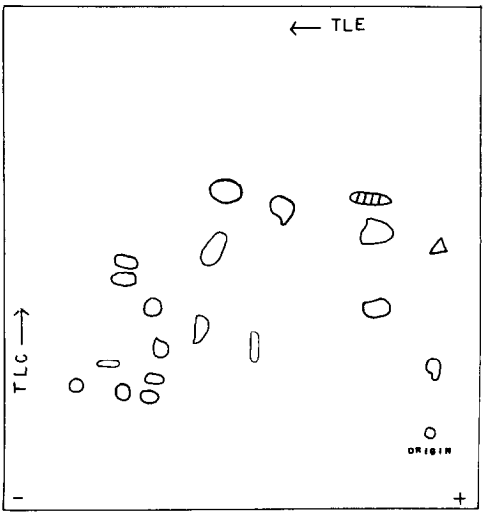

Figure 4. A peptide map of the major radioactive fraction (1 ml fractions 77 through 100) from the Sephadex G-25 column. These fractions were pooled and lyopholized. The residue was dissolved in 10% isopropanol-water and three nanamoles applied to one corner of a cellulose sheet (Eastman). Thin-layer electrophoresis (TLE) in one dimension (pyridine-acetic acid-water, 1:10:289, v/v) and thin-layer chromatography in the second dimension (isoamyl alcohol-pyridine-water-ethanol-acetic acid, 70:70:60:20:5, v/v) was performed (9). The map was visualized under ultraviolet light after spraying with fluorescamine, 15 mg/ml acetone followed by 0.2M sodium borate, pH 8.0 and heating to 60°C for 20 minutes. The spots were cut into 10 ml of INSTAGEL and counted.

Results and Discussions

The inactivation of aconitase by phenacyl bromide is first order in enzyme sites (Fig. 1) and first order in reagent, one mole of phenacyl bromide reacting with one mole of enzyme (Fig. 2) at complete inactivation. The rate of inactivation is inhibited by the competitive inhibitor tricarballylate (Fig. 1) and by the substrate, citrate (Fig.3). For the latter experiment active enzyme was used and Figure 3 presents a plot of reciprocal first order slopes of inactivation against citrate concentration. As expected for substrate inhibition of reaction with an active site reagent, the plot is linear, and conforms to the readily derived equation (1). K_{cit} calculated from the intercept and slope has a value of 0.14mM,

$$\frac{1}{k'} = \frac{1}{k_o'} + \frac{\text{citrate}}{k_o' K_{cit}} \qquad (1)$$

a value of the same order of magnitude as the K_M, 0.22mM determined at 20° under the same conditions.

Determination with Ellman's reagent in urea of thiol equivalents of untreated enzyme and phenacylated enzyme leads to identification of a cysteine residue as the site of reaction, a difference of 1.30 thiophenolate equivalents/mole being found between untreated (9.95 equivalents/mole) and phenacyl-enzyme (8.65 equivalents per mole). In the absence of urea, reaction of Ellman's reagent with both aconitase and phenacylated-aconitase is slow and incomplete, phenacylation having no apparent effect on conformation.

Paper chromatography, detailed in the Methods section, of an acid hydrolysate of [^{14}C] phenacyl-aconitase, one [^{14}C] phenacyl residue incorporated per mole, corroborates assignment of a cysteine residue as the site of phenacylation. Only one radioactive peak (R_f 0.63) appears, this spot coincident with the only UV absorbing spot and identical to authentic [^{14}C] phenacyl-L-cysteine synthesized in our laboratory.

The fractionation of tryptic peptides of tricarballylate-free [^{14}C] phenacyl, reduced and carboxymethylated aconitase was accomplished on a 1.5 x 84 cm column of Sephadex G-25 equilibrated with 0.05M ammonium bicarbonate. The major radioactive fraction, associated with lower molecular weight peptides, contained 90% of the applied counts. Peptide mapping of the pooled fractions, using fluorescamine for detection, revealed 19 fluorescent spots in which only one coincided with the [^{14}C] counts (cross-hatching in Figure 4, 914 cpm). Since tricarballylate does not completely inhibit phenacylation (Fig. 1) an experiment was performed on aconitase that was phenacylated in the presence of tricarballylate. Identical results, the radioactivity being on the same peptide, were obtained.

Aconitase, then, is inactivated by a 1:1 reaction with phenacyl bromide, a single active site thiol being alkylated.

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REFERENCES

1. Glusker, J.P. (1971) The Enzymes, 3rd. edit., 5, 413-439.
2. Kennedy, Sr. M.C., Rauner, R., and Gawron, O. (1972) Biochem. Biophys. Res. Comm. 47, 740-745.

3. Gawron, O., Kennedy, Sr. M.C., and Rauner, R. (1974) Biochem. J. 143, 717-723.
4. Villafranca, J.J. and Mildvan, A.S. (1971) J. Biol. Chem. 246, 3454-3463.
5. Gawron, O., Waheed,A., Glaid, A.J. III, and Jaklitsch, A. (1974) Biochem. J. 139, 709-714.
6. Cowper, R.M. and Davidson, L.H. (1943) Organic Syntheses (A.H. Blatt, ed.) Coll. Vol. II, p. 480, John Wiley & Son, New York.
7. Vogel, A.I. (1946) Practical Organic Chemistry, P. 693, Longman, Green and Company, New York.
8. Glasel J.A., Vratsanos, S.M.,Wasserman, N. and Erlanger, B.F. (1966) Arch. Biochem. Biophys., 115, 237-246.
9. Rauner, R. Ph.D. Dissertation (1972) Duquesne University, Pittsburgh, Pa.