Properties of Pig Heart Aconitase

By OSCAR GAWRON, Sr., MARY C. KENNEDY and RICHARD A. RAUNER
Department of Chemistry, Duquesne University, Pittsburgh, Penn. 15219, U.S.A.

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Comparison of pig heart aconitase (Kennedy *et al.*, 1972) with yeast (*Candida lipolytica*) aconitase (Suzuki *et al.*, 1973) reveals similarities in molecular weight and iron content but not in sulphide content. Comparison with the Mildvan & Villafranca (1971) pig heart aconitase preparation reveals differences in iron ligands, specific activity and other properties; these differences possibly arise from protein association as pig heart protein associates under a variety of conditions. The electron spin resonance spectrum, g 4.25, and the low molar relaxivity, $473\text{ M}^{-1}\cdot\text{s}^{-1}$, of water H^+ suggest the presence of high-spin Fe(III) unco-ordinated to water in the enzyme. The iron chromophore on acid titration at 320nm gives a curve with an inflexion at pH 4.2. Ten of 16 expected thiol equivalents are titrated with *p*-hydroxymercuribenzoate suggesting the presence of cystine as well as cysteine residues. Inhibition of the activation of inactive (activatable) enzyme is sigmoidally related to the molar ratio, *p*-hydroxymercuribenzoate/enzyme with 10–11 mol of mercurial compound causing complete inhibition. Active enzyme, free from activating reagents, requires high molar ratios of mercurial compound for rapid inhibition. In terms of *p*-hydroxymercuribenzoate the enzyme then lacks an essential thiol group.

The aconitase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3]-catalysed reactions have long been under investigation with respect to stereochemistry and mechanism (Dickman, 1961; Glusker, 1971). Although the stereochemistry of the substrates, *cis*-aconitate, D_5 -*threo*-isocitrate and citrate (Gawron & Glaid, 1955; Gawron *et al.*, 1961; Kaneko & Katsura, 1960; Kaneko *et al.*, 1960; Hanson & Rose, 1963; Glusker *et al.*, 1958; Patterson *et al.*, 1962) defines the stereochemistry of the inter-conversions, mechanistic explanations of reversible replacement of H^+ by OH^- with retention of configuration and H^+ (Gawron *et al.*, 1961; Speyer & Dickman, 1956; Rose & O'Connell, 1967; Gawron & Mahajan, 1966; Hendrickson & Srere, 1968; Glusker, 1968; Glusker, 1971; Villafranca & Mildvan, 1972) remain to be experimentally demonstrated.

The above investigations have been conducted, in the main, with pig heart aconitase and, surprisingly, with relatively crude preparations of the enzyme. To remedy this situation and to provide pure enzyme for structural characterization as well as for further mechanistic investigation, we undertook several years ago extension of the Morrison (1954) procedure for purification of pig heart aconitase. Inclusion of an ion-exchange step in the Morrison procedure and final purification by isoelectric focusing yielded homogeneous enzyme (Kennedy *et al.*, 1972) in contrast with the approx. 75% pure enzyme obtained previously. The present paper describes several of the molecular and chemical properties of the pure enzyme.

It may also be noted that investigations (Gawron *et al.*, 1974*a,b*) with the pure enzyme have demonstrated that it has only one active site/mol, this value not being able to be determined with impure enzyme (Glusker, 1971).

Experimental

NADP^+ , L-ascorbate, Hepes [2-*N*-2-hydroxyethylpiperazin-*N'*-yl]ethanesulphonic acid], isocitrate dehydrogenase, cysteine hydrochloride and sodium *p*-hydroxymercuribenzoate solution were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Tricarballic acid was obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A., triethanolamine hydrochloride (heavy metal-free) was from Calbiochem, Los Angeles, Calif., U.S.A., and Sephadex gels were from Pharmacia, Piscataway, N.Y., U.S.A. Ferrous ammonium sulphate hexahydrate (reagent-grade) was obtained from Fisher Chemical Corp., Pittsburgh, Pa., U.S.A.

Aconitase was prepared as described (Kennedy *et al.*, 1972) and individual 0.5 ml portions containing 2–10 mg of enzyme were stored at -20°C . The enzyme was activated and then assays were carried out at 26°C by the method of Rose & O'Connell (1967) in 0.01 M-triethanolamine chloride buffer, pH 8.0. Enzyme was activated by the method of Rose & O'Connell (1967) or by the method of Gawron *et al.* (1974*a*) with activating solution, pH 7.5, containing 30 mM-ascorbic acid (neutralized to pH 7.5 with Tris)

10mM-cysteine and 5mM-ferrous ammonium sulphate. Specific activities are reported as μmol of NADPH/min per mg of protein, protein concentrations being determined from $E_{1\text{cm}}^{1\%} = 11.5$ at 280nm (Kennedy *et al.*, 1972). Recent preparations show specific activities of 12.6, 11.1, 12.0 and 12.7. Addition of 81.6 μl of 4M-(NH_4)₂SO₄ in 15mM-Tris-tricarballylate, pH 6.2, to 50 μl of enzyme solution (27.5 mg/ml in the same buffer) results in the formation of micro crystals after storage at room temperature (23°C) for several weeks. Active enzyme free from activating reagents was prepared by the method of Gawron *et al.* (1974a). Tris-tricarballylate buffers were prepared by titrating tricarballic acid solutions of known concentration to the desired pH with solid Tris and diluting to the final concentrations with a small amount of water.

Thiol residues were determined by titration with *p*-hydroxymercuribenzoate (Boyer, 1954; Benesch & Benesch, 1962). Extinction values after addition of titrant remained constant over 5 min. Subsequently, extinction values increased owing to turbidity. Reductive carboxymethylation was carried out by the procedure of Keresztes-Nagy *et al.* (1969). Protein hydrolysates were prepared by heating samples with 6M-HCl at 110°C for 24 h in sealed evacuated tubes. Amino acids were determined by conventional one-column techniques by using the Technicon analytical system (Technicon Instruments Corp., Tarrytown, N.Y., U.S.A.).

The e.s.r. (electron spin resonance) spectrum of aconitase was determined at 77°K with the Varian E-246 quartz Dewar attachment for the Varian E-4 e.s.r. spectrometer. The effect of aconitase on the relaxation rate of water protons was measured in an N.M.R. Specialities PS60W-pulsed n.m.r. (nuclear magnetic resonance) spectrometer at 24.3 MHz. In both instances the procedures of Villafranca & Mildvan (1971) were used.

Ultracentrifugation studies were carried out by the method of Yphantis (1964) with the Beckman model E analytical ultracentrifuge (Spinco Division, Beckman Instruments Inc., Palo Alto, Calif., U.S.A. equipped with interference optics. The method of Andrews (1970) was used for estimation of molecular weight by gel filtration; a column (2.5 cm $\times$ 75 cm) of Sephadex G-200 was used with 0.05M-Tris-HCl-0.1M-KCl buffer, pH 7.5, as eluent (Andrews, 1964, 1965).

The Beckman DU spectrophotometer with Gilford Instrument Laboratories (Oberlin, Ohio, U.S.A.) modifications, expanded range recorder and constant-temperature cell compartment was used for spectrophotometric measurements.

Results and Discussion

Comparison (Table 1) of pig heart aconitase with the yeast (*C. lipolytica*) aconitase (Suzuki *et al.*, 1973)

Table 1. Comparison of pig heart and yeast aconitase

Data for pig heart aconitase are from Kennedy *et al.* (1972) and from this paper. Data for yeast (*C. lipolytica*) aconitase are from Suzuki *et al.* (1973). The sulphide content of pig heart aconitase was determined by the method of Lovenberg *et al.* (1963), the pH after precipitation of Zn(OH)₂ not exceeding 7.5.

Property	Pig heart	Yeast
Mol.wt.	66000	68000
g-atom of Fe/mol	2	2
S ²⁻ /mol (acid-labile)	3	1
pI	8.5	4.2
Crystal form	Needles	Needles
Reconstitution	—	From apoprotein

Fig. 1. Association of aconitase

Enzyme was dialysed for 36 h against 0.05M-Tris-HCl, pH 7.5, then stored at -20°C for 10 days. Sedimentation conditions were 10000 rev./min at 10.1°C and enzyme concentration was 1.0 mg/ml. The apparent molecular weights were 180000 and 280000.

reveals similarities in molecular weight, iron content and crystal habit but differences in sulphide content and pI. In addition the yeast enzyme is presumably isolated as fully active enzyme, whereas the heart enzyme requires an additional g-atom of iron/mol to activate it (Gawron *et al.*, 1974a). The presence of sulphide in addition to iron suggests that these aconitases may be non-haem iron-sulphur proteins lacking the usual 1:1 iron/sulphide ratio.

The heart enzyme associates under a variety of conditions. Storage at -20°C of enzyme frozen after dialysis against 0.05M-Tris-HCl, pH 7.5, resulted in a noticeable diminution of brown colour. After 10 days at -20°C, the enzyme was completely inactive and gave evidence (Fig. 1) for molecular-weight species of 180000 and 280000 on ultracentrifugation. In another experiment dialysed enzyme stored for 5 days at -20°C yielded evidence (Fig. 2) for dimeriza-

Fig. 2. Dimerization of aconitase

Enzyme was dialysed against 0.05 M-Tris-HCl, pH 7.5, for 36h, then stored at -20°C for 5 days. Enzyme solution (0.5ml; 4.0mg/ml) diluted with 2.5ml of eluting buffer, was submitted to gel filtration on Sephadex G200 at 23°C and 3.0ml fractions were collected. Molecular weights were 115000 and 63000.

tion. Since the enzyme is stable on storage at -20°C in the presence of tricarballoylate, a competitive inhibitor (Gawron & Birckbichler, 1971; Fansler & Lowenstein 1969), 15 mM-Tris-tricarballoylate, pH 7.8, being used, it is possible that replacement of tricarballoylate by Cl^{-} underlies the inactivation. This point, however, has not been investigated further.

The enzyme also associates at pH4 and 2°C , gel filtration at this pH (in acetate buffer) revealing an associated species of mol.wt. 280000, suggestive of a tetramer. Conditions usually used for disclosing subunit structure also lead to association. Reductive carboxyamidomethylation in guanidinium chloride (Fish *et al.*, 1969) or in 1% (w/v) sodium dodecyl sulphate (Kennedy, 1972) and maleylation (Bruton & Hartley, 1968) yielded only associated species (Kennedy, 1972). These results prevent comparison with the related tricarboxylic acid-cycle enzyme, succinate dehydrogenase, a non-haem iron-sulphur protein with a subunit structure (Davis & Hatefi, 1971).

Association of ferredoxin, a non-haem iron-sulphur protein is attributed to thiol-disulphide exchange with the necessary disulphide being formed by aerobic oxidation (Gersonde *et al.*, 1971). Heart aconitase may associate by a similar mechanism as present evidence (given below) indicates the presence of cysteine and cystine residues in the enzyme. In this connexion enzyme samples, stored at -20°C after dialysis against Tris-HCl buffer, undergo gelation on heating to 40°C (Kennedy, 1972). Similar behaviour is noted with serum albumin, thiol-disulphide exchange being responsible for the polymerization (Huggins *et al.*, 1951; Reithel, 1963; Bresler, 1971).

The iron of aconitase exists in oxidation state III, the e.s.r. spectrum (Fig. 3) with g 4.25, being similar

to those of ferric transferrin, g 4.1, ferric conalbumin, g 4.1 (Blumberg, 1967) and rubredoxin, g 4.3 (Peisach *et al.*, 1971); rubredoxin is a sulphide-lacking non-haem iron-sulphur protein. The presence of iron (III) in aconitase may also account for the lowered p -hydroxymercuribenzoate titre at pH4.6, 8 equivalents compared with 10 equivalents at pH 7.0 (Fig. 4); the stoichiometry of Fe^{3+} interaction with thiol or sulphide is:

which accounts for the disappearance of 2 p -hydroxymercuribenzoate equivalents/mol.

Fig. 3. Difference e.s.r. spectrum (derivative of absorption against magnetic field) of aconitase (5.3mg/ml) after subtraction of buffer spectrum

Scan time, 4min; time constant, 3s; gain, 2.5×10^3 microwave power, 200W; microwave frequency, 9.181 GHz; $T = 77^{\circ}\text{K}$.

Fig. 4. Titration of enzyme with p -hydroxymercuribenzoate

Titrant, 0.454mM, 364 μg of enzyme in 2.0ml of 0.1M-sodium phosphate-6M-urea buffer pH7.0; $\blacktriangle$, 400 μg of enzyme in 2.0ml of 0.3M-sodium acetate-acetic acid-6M-urea, pH4.6; extinctions were measured at 250nm and 255nm respectively at 23°C in 1cm-light-path silica cuvettes.

Fig. 5. Spectrum of aconitase (0.8 mg/ml) at several pH values

Enzyme in 15 mM-Tris-tricarballylate, pH 7.5, adjusted to the required pH value with NaOH or HCl; ----, pH 9.5; —, pH 7.5; — · —, pH 3.95.

The optical spectrum of aconitase at neutral pH (Fig. 5), although resembling that of ferric transferrin (Aasa *et al.*, 1963), shows considerable absorbance over the 315–460 nm region, a region in which the iron chromophores of non-haem iron-sulphur proteins, both natural and synthetic, show discrete absorbance bands (Yang & Huennekens, 1969). For these proteins ϵ values/iron atom are in the range 5800–7200 at 318 nm and 3900–4600 at 315 nm. For aconitase at pH 7.5 the ϵ value/iron atom is 7600 at 318 nm. The iron chromophore of aconitase is acid-sensitive (Fig. 5) and spectrophotometric titration at 320 nm reveals (Fig. 6) a titration curve with an inflexion at pH 4.2. However, the titration is not reversible and addition of base to pH 7 causes turbidity. Presumably the protein undergoes association during the titration as the data at pH 4.0 indicate. Readjustment of the pH to 7 may then result in precipitation. Of further interest is the levelling of the extinction at 320 nm to approximately one-half of the original value. This may indicate different binding characteristics of each of the two iron atoms of inactive aconitase.

In connexion with the iron chromophore, the low value ($473 \text{ M}^{-1} \cdot \text{s}^{-1}$) found (J. J. Villafranca, personal communication) for the longitudinal molar relaxivity of water protons in the presence of the enzyme is indicative of the absence of water co-ordinated to the Fe (III) atoms of aconitase. Such low values of the molar relaxivity are also considered to be typical of non-haem iron-sulphur proteins (Mildvan & Cohn, 1970), all ligands being sulphur ligands. Whereas the sulphide and cysteine content and molar-relaxivity data are consistent with only sulphur ligands for the iron atoms of aconitase, the resemblance of the optical spectrum to ferric transferrin is not. In addition, the titration curve at 320 nm (Fig. 6) with inflexion at pH 4.2 may be due to the involvement of a carboxyl

group. Considerations of structure must therefore await further evidence. It is noteworthy, however, that the two-iron cluster structure (Orme-Johnson, 1973) can accommodate either three cysteine, two sulphide and one cysteine persulphide ligands, or four cysteine, one sulphide and one persulphide ligands. With respect to the latter, an -Fe-S-S-Fe bridge has been found in $[\text{CpFe}(\text{SC}_2\text{H}_5)_2\text{S}]_2$ (where Cp is cyclopentadienyl) (Kubas *et al.*, 1973).

The low value ($473 \text{ M}^{-1} \cdot \text{s}^{-1}$) found for the molar relaxivity of water protons in the presence of pig heart aconitase prepared by the method of Kennedy *et al.* (1972) contrasts with the value, ($5900 \text{ M}^{-1} \cdot \text{s}^{-1}$) found for the Villafranca & Mildvan (1971) preparation. The latter value indicates the presence of 3 ± 1 water molecules in the inner co-ordination sphere of the Fe(III) (Villafranca & Mildvan, 1971). In comparison with the enzyme of Kennedy *et al.* (1972), it would seem, then, that several iron ligands are replaced by water molecules in the course of the Villafranca & Mildvan (1971) procedure. The latter preparation also demonstrates higher molecular weight (89000), lower iron content (1 g-atom/89000 g) and lower specific activity (6–9) (J. J. Villafranca, personal communication) than the enzyme of Kennedy *et al.* (1972). These properties are consistent with associated enzyme and it is noteworthy that association of ferredoxin results in lowered activity, loss of iron and loss of sulphide (Gersonde *et al.*, 1971).

Some 10 thiol equivalents/mol are revealed by titration with mercurial compound in 6M-urea solu-

Fig. 6. Spectral titration at 320 nm

Enzyme solution (0.4 ml; 2.5 mg of enzyme) diluted with 2.0 ml of 0.01M-HCl was titrated (by micro-burette) with 0.100M-HCl. E_{320} values were corrected for volume changes; at 23°C in 1 cm light-path silica cuvettes.

Fig. 7. Effect of *p*-hydroxymercuribenzoate on aconitase activity

Portions (190 μ g) of inactive enzyme in 50 μ l of buffer were treated at 23°C with incremental volumes of 6.3 μ l of 0.5 mM-compound in 0.1 M-sodium phosphate, pH 7.0, for 3 min (complete reaction) then activated by modification of the method of Rose & O'Connell (1967), cysteine being replaced by 90 mM-ascorbate, pH 7.5.

Fig. 8. Effect of *p*-hydroxymercuribenzoate at 25°C on aconitase activity

Active enzyme (72.2 μ g; 1.1 nmol) in 100 μ l of 15 mM-Tris-tricarballoylate was treated with 200 μ l of 150 mM-sodium-phosphate, pH 7.0, containing various amounts of mercurial compound: ●, control; ■, 4.54 nmol; ▲, 9.08 nmol; ▲, 15.8 nmol.

tion (Fig. 4) as well as in aqueous solution. Reductive carboxymethylation yields 10 *S*-carboxymethyl-cysteine residues/mol (Rauner, 1972). Since 16 equivalents/mol would be expected (Petering & Palmer, 1970) on the basis of ten cysteine residues plus three sulphide groups/mol, the results of *p*-hydroxymercuribenzoate titration indicate the presence of three cysteine residues.

On the basis of qualitative evidence the enzyme has long been considered thiol enzyme (Dickman, 1961; Morrison, 1961). Corroborating Morrison's (1961) results, iodoacetate and iodoacetamide cause, under experimental conditions similar to those of Morrison, only slight inhibition whereas *p*-hydroxymercuribenzoate markedly inhibits the enzyme (Figs. 7 and 8). Inactive (activatable) enzyme, activated with Fe^{2+} and ascorbate after reaction with the mercurial compound, gives a sigmoidal relationship (Fig. 7) between activity remaining and equivalents of mercurial compound added. These results in terms of *p*-hydroxymercuribenzoate reactivity, do not indicate the presence of an essential thiol group. The co-operativity of the inhibition does suggest denaturation as the cause for inhibition. With active enzyme, inactivation with mercurial compound is time-dependent (Fig. 8) and a considerable molar excess is necessary for marked inhibition in a relatively short period. An essential thiol group in terms of *p*-hydroxymercuribenzoate and iodoacetate reactivity is therefore not present in inactive or active aconitase, and inactivation by mercurial compound of both inactive and active enzyme must be considered to be due to a denaturative alteration of the structure of the protein. These results, however, do not preclude the participation (Glusker, 1971) of a single thiol group in the catalytic mechanism since other thiol reagents may manifest sufficient differential reactivity to indicate an essential thiol group.

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