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Nitro Analogues of Citrate and Isocitrate as Transition-State Analogues for Aconitase†

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ABSTRACT: Nitro analogues of citrate (2-hydroxy-3-nitro-1,2-propanedicarboxylic acid) and isocitrate (1-hydroxy-2-nitro-1,3-propanedicarboxylic acid) are carbon acids with pK values of 10.80 and 9.46, respectively. Both compounds are competitive inhibitors of aconitase with their affinity for the enzyme dependent on the ionization state of the carbon acid. When fully ionized, the citrate and isocitrate analogues have K_i values of 59 nM and 72 nM, respectively, which increase to 1.7 mM and 58 μM upon protonation of the carbon acid. In addition to the pH-dependent change in its affinity for aconitase, the isocitrate analogue (but not the citrate analogue) exhibits a slow time-dependent increase in its affinity for enzyme, with an equilibrium constant for the change of 105 and a final K_i of 680 pM for the ionized form and an equilibrium constant of 83 and a final K_i of 680 nM for the protonated form. The rate constant for formation of the tight complex is 6.6 min^{-1}, and that for the reverse isomerization is 0.063 min^{-1}. The Michaelis constants for citrate (160 μM) and isocitrate (49 μM) are 2700 and 72 000 times greater than the final K_i values for the ionized nitro analogues of citrate and isocitrate, respectively. We believe the tight binding of the nitro compounds is due to their similarity in structure to carbanion intermediates in the reaction (bound as aci-acids) which are tetrahedral at the carbon bearing a hydroxyl and trigonal at the carbon lacking a proton.

A renewed interest in the mechanism of action of aconitase (EC 4.2.1.3) has ensued with the realization that the enzyme isolated from beef heart mitochondria is a high-potential iron–sulfur protein (Ruzicka & Beinert, 1978; Kurtz et al., 1979). As reductants other than Fe^{2+} can serve to activate the enzyme (Ruzicka & Beinert, 1978; Schloss, 1979), the long-held role of Fe^{2+} in the activation of aconitase has come into question, and the proposed ferrous wheel mechanism is suspect [for a review of this and other proposed mechanisms, see Glusker (1971)]. Additional support for the absence of a role for Fe^{2+} other than reduction of the iron–sulfur cluster comes from the observation that aconitase can be isolated anaerobically from pig liver cytosol to yield a fully active enzyme (Eanes & Kun, 1974).

While mechanistic details of aconitase action are scanty, it appears that the enzyme plays a role as base, abstracting a proton from citrate or isocitrate (Rose & O'Connell, 1967). The general conclusion that the enzyme proceeds by a carbanion mechanism is not universally accepted, however, as Thomson et al. (1966) proposed a carbonium ion mechanism on the basis of the relatively small primary isotope effects observed with deuterated substrates. In the course of further mechanistic studies of aconitase, we have found that the fully ionized forms of nitro analogues of citrate and isocitrate (which can be considered analogues of carbanion reaction intermediates) are potent inhibitors of the enzyme, and the present report deals with the properties of this inhibition.

Experimental Procedures

Materials. *threo*-DL-Isocitrate, *cis*-aconitate, Mes,[1] Hepes, Tris, Caps, *cis*-oxalacetic acid, and glyoxylic acid were from Sigma Chemical Co. *N*-Ethylmorpholine, ethanolamine, and 3-nitropropionic acid were from Aldrich. The concentration of *threo*-D$_S$-isocitrate in stock solutions of *threo*-DL-isocitrate was determined with isocitrate dehydrogenase by the method of Rose & O'Connell (1967); all references to isocitrate refer to *threo*-D$_S$-isocitrate. *threo*-L$_S$-Isocitrate is a poor inhibitor of aconitase (K_i > 4 mM; Villafranca, 1972; Thomson et al.,

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[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Caps, 3-(cyclohexylamino)-propanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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1966; Henson & Cleland, 1967) and its presence was ignored.

Buffers used in kinetic studies (chosen in part due to their low absorbance at 240 nm) included acetate (pH 4.5, 5.0, and 5.5), Mes (pH 5.5, 6.0, and 6.5), Hepes (pH 7.0, 7.5, and 8.0), *N*-ethylmorpholine (pH 7.0, 7.5, and 8.0), Tris (pH 8.0, 8.5, and 9.0), ethanolamine (pH 9.0, 9.5, and 10.0), and Caps (pH 10.0, 10.5, and 11.0). All buffers were used at 0.1 M, with the pH adjusted with NaOH or HCl.

Aconitase was partially purified from beef liver by the method of Henson & Cleland (1967). The enzyme preparation had a specific activity of 0.81 unit/mg when activated and assayed by the method of Rose & O'Connell (1967). Without activation the enzyme retained a specific activity of 0.22 unit/mg, and this unactivated enzyme was used for the kinetic studies reported here.

General Methods. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. Melting points were taken with a Mel-Temp apparatus and are uncorrected. NMR spectra were obtained with a 60-MHz Varian A60 spectrometer. Spectra were recorded from samples dissolved in D_2O with sodium 2,2-dimethyl-2-silapentane-5-sulfonate used as an internal reference. Thin-layer chromatography was conducted on glass plates coated with silica gel containing a fluorescent indicator (silica gel 60 F-254 from E & M Laboratories). Solvents used were 1-butanol–acetic acid–H_2O (50:25:40) and ethanol–H_2O (1:1). Nitro compounds were detected by spraying chromatograms with 5 N KOH, followed by examination under ultraviolet light.

2-Hydroxy-3-nitro-1,2-propanedicarboxylic Acid. To 5 g of *cis*-oxalacetic acid (37 mequiv) and 90 mequiv of NaOH in 50 mL of water was added 16.5 g of nitromethane (0.27 mol). After stirring the heterogeneous mixture at room temperature for 30 min, we adjusted the pH to 7.0 with 6 N HCl. Excess nitromethane was removed by extraction with two 100-mL portions of ethyl ether. The aqueous phase was diluted to 1 L with H_2O and applied to a Dowex AG1-X4 (3 × 30 cm) column in the chloride form. After washing the column with 500 mL of water, we eluted the product with a 0–1 M KCl gradient (2 L). Fractions (6 mL) containing product were pooled (320 mL), and the pH was adjusted to 1.8 with 6 N HCl. Water was removed by rotary evaporation. The white solid was dried by suspending it twice in 50 mL of absolute ethanol and removal of the ethanol–water by rotary evaporation. The solid was then extracted with two 100-mL portions of ethyl ether. The extract was concentrated to 15 mL to which an equal volume of benzene was added. Crystals formed at 5 °C overnight, yielding 4.7 g of a white powder (66% yield): mp 130–131 °C dec; ^{1}H NMR δ_B 2.87, δ_A 3.06 (J_{AB} = 17 Hz, two AB patterns $-CH_2CO_2H$), δ_B 4.90, δ_A 5.10 (J_{AB} = 13 Hz, $-CH_2NO_2$). Anal. Calcd for $C_5H_7NO_7$ (193.13): C, 31.10; H, 3.65; N, 7.25; O, 58.0. Found: C, 31.24; H, 3.75; N, 7.25; O, 57.93. The material exhibited two acid pK values of 4.45 and 2.66 with an equivalent molecular weight of 99 (calcd M_r 96.5). The product was homogeneous as judged by thin-layer chromatography with butanol–acetic acid–H_2O (R_f 0.19) or ethanol–H_2O (R_f 0.73) as the solvent. In 0.1 N KOH an ϵ_{239} = 9600 M^{-1} cm^{-1} (λ_{max}) was observed for the 3-carbanion.

1-Hydroxy-2-nitro-1,3-propanedicarboxylic Acid Bis(cyclohexylammonium salt). To 20 mL of deionized water 3-nitropropionic acid (2.4 g, 20 mmol) and glyoxylic acid (1.8 g, 20 mmol) were added, and the final pH was adjusted to 9.0 with 10 N KOH. After 3 days at room temperature the pH was adjusted to 1 with 6 N HCl and the solution was treated with Norit. The mixture was filtered and the filtrate was taken to dryness by rotary evaporation. The residue was extracted with two 20-mL aliquots of 50% ethanol–ethyl ether. The combined extracts were concentrated to dryness by rotary evaporation and the residue was dissolved in 30 mL of ethyl ether. This material was applied to a 3 × 50 cm silica gel column (Baker; 60–200 mesh). The product was eluted with ether and pooled (50 mL). The ether was removed by rotary evaporation and the residue was dissolved in 50 mL of deionized H_2O. The pH was adjusted to 6 with cyclohexylamine and the water was removed by rotary evaporation. The residue was dissolved in a minimal volume of hot ethanol and allowed to crystallize at −10 °C, yielding 4.8 g of a white powder (61% yield): mp 160 °C dec; ^{1}H NMR (superimposed upon the spectrum of cyclohexylamine) δ_B 2.33, δ_A 3.00, δ_X 5.23 (J_{AB} = 18 Hz, J_{AX} = 11 Hz, J_{BX} = 3 Hz, $HO_2CCH_2CHNO_2$), δ_Y 4.60 (J_{XY} = 3 Hz, $HO_2CCHOH-$). Anal. Calcd for $C_{17}H_{33}N_3O_7$ (391.47): C, 52.16; H, 8.50; N, 10.73; O, 28.61. Found: C, 51.98; H, 8.58; N, 10.63; O, 28.54. The material exhibited two acid pK values of 2.58 and 3.89 with an equivalent molecular weight of 200 (calcd M_r 196). The product was homogeneous (R_f 0.44) as judged by thin-layer chromatography with butanol–acetic acid–H_2O as the solvent. In 0.1 N KOH an ϵ_{233} = 10000 M^{-1} cm^{-1} (λ_{max}) was observed for the 2-carbanion.

The nitro analogue of citrate used in these studies is presumably an equal mixture of its two enantiomers. Similarly, the isocitrate analogue contains an undetermined amount of each of the four possible optical isomers (although the configuration at C-2 will be equilibrated when appreciable carbanion is present). It is likely that 2(*R*)-hydroxy-3-nitro-1,2-propanedicarboxylic acid and 1(*R*)-hydroxy-2(*S*)-nitro-1,3-propanedicarboxylic acid are the potent inhibitors of aconitase. The nitro analogue of citrate is a substrate for citrate lyase (D. J. T. Porter, unpublished experiments), which cleaves half of the racemic compound to acetate and 3-nitropyruvate. At pH 9.5 under conditions where control samples of the citrate analogue inhibited aconitase 60%, those treated with citrate lyase showed no inhibition. In view of the stereochemistry of the citrate lyase reaction (Srere, 1975) and the mode of cleavage of the nitro compound, these results confirm that it is the 2(*R*) isomer of the citrate analogue which in the carbanion form is strongly inhibitory vs. aconitase.

Stability of the Nitro Analogues. The nitro analogues of citrate and isocitrate are stable at low pH but decompose by a base-catalyzed process ($\sim 4 \times 10^{-4}$ min^{-1} at pH 8.1 and room temperature) which between pH 8 and pH 10 increases in rate by a factor of 10 per pH unit. This breakdown, which apparently results from decomposition of the alkoxide form of the analogue, gives oxalacetate (followed with malate dehydrogenase) and nitromethane from the citrate analogue and glyoxylate (followed with lactate dehydrogenase) and nitropropionate from the isocitrate analogue. When the citrate analogue is dissolved in 0.1 N base, alkoxide formation (with subsequent breakdown) and carbanion formation compete, and, after reneutralization and analysis for oxalacetate, it appears that 25% breakdown has occurred. The carbanion is stable once formed, however, so the time spent at pH 13 does not alter the degree of breakdown. The breakdown is also greatly accelerated by metal ions, presumably by formation of chelates with the alkoxide form. At pH 8.1, 10 mM Mg^{2+} causes the citrate analogue to decompose at room temperature at a rate of ~ 0.06 min^{-1}. In all of the studies reported here, the rate of breakdown of the nitro analogues was not sufficient to cause any problems.

Assays. Assays were conducted at 25 °C by monitoring aconitate at 240 nm, utilizing the appropriate extinction

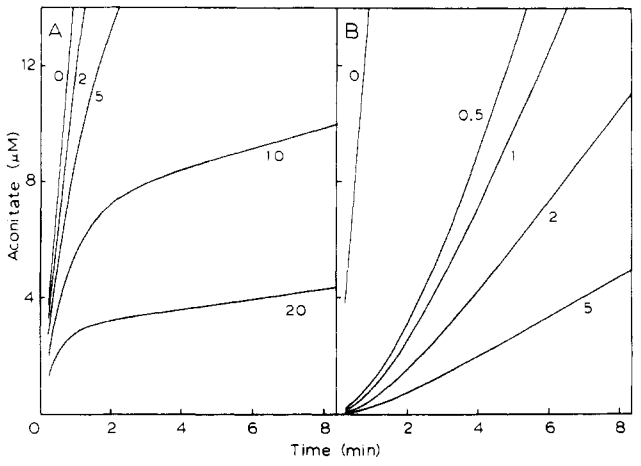


FIGURE 1: Assay time courses in the presence of the nitro analogue of isocitrate (0.1 M Tris, pH 8.0). (A) Assays were initiated with enzyme. (B) Assays were initiated with 525 μM isocitrate, the same substrate concentration used in (A), after preincubating aconitase with the nitro analogue of isocitrate for 30–60 min. The micromolar concentrations of the analogue used are designated in the figure.

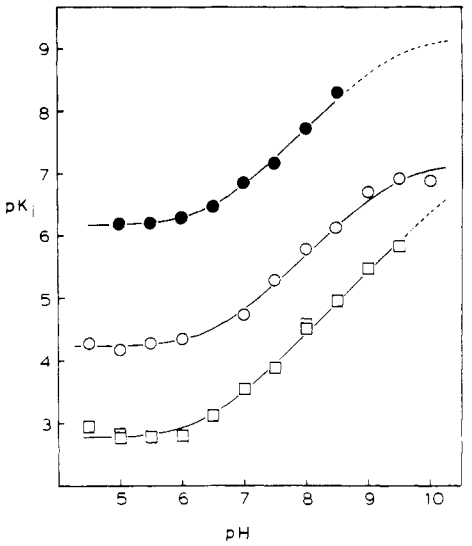


FIGURE 2: Variation of pK_i with pH. The pK_i profiles are shown for the nitro analogue of citrate (□), for the initial rate inhibition (○), and for the final steady-state inhibition (●) by the nitro analogue of isocitrate.

coefficient of aconitate at the assay pH (Schloss, 1979). Fe^{2+}-activated enzyme was not used when monitoring aconitate at 240 nm due to the time-dependent, UV-absorbing complex formed between iron and citrate (Villafranca, 1974; Henson & Cleland, 1967) and the known effect of divalent cations on the equilibrium constant of aconitase (Blair, 1969) and its kinetic constants.

Data Processing. Kinetic data were fitted to appropriate rate equations by the least-squares method, using FORTRAN programs which assume equal variance for the velocities or the logarithm of the fitted parameter (Cleland, 1967). Data from experiments with the nitro analogues as competitive inhibitors were fitted to eq 1 and 2, where v is the experimentally determined velocity, V is the maximum velocity, A is the substrate concentration, K is the Michaelis constant, K_{is} is the slope inhibition constant, and K_{ii} is the intercept inhibition constant.

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \quad (1)$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (2)$$

$$\log K_i = \log \left[\frac{K_{iL}}{1 + (K'/[H^+])} \right] \quad (3)$$

$$P = P_0 + (v_i - v_f)/k + v_f t + (v_f - v_i)e^{-kt}/k \quad (4)$$

The K_i values of the citrate and isocitrate analogues as a function of pH were fitted to eq 3, where K_i is the inhibition constant at the experimental pH, K_{iL} is the inhibition constant for the inhibitor at low pH, and pK' is the pH at which the K_i value begins to decrease with pH. Since $K' = K_3K_{iL}/K_{iH}$, where pK_3 is the pK of the inhibitor for ionization to a carbanion and K_{iH} is the inhibition constant of the fully ionized form, K_{iH} can be calculated from K', K_3, and K_{iL}.

Assay progress curves in the presence of the isocitrate analogue, which exhibited a burst or lag when the assay was initiated with enzyme or substrate, respectively, were fitted to eq 4, where P is the product produced at time t, v_i is the initial velocity, v_f is the final steady-state velocity, k is the first-order rate constant for the burst or lag, and P_0 is the product present at zero time. Initial estimates of k, necessary in fitting data to eq 4, were made by drawing tangents to initial and final slopes of assay progress curves. The time coordinate of the intersection point of these lines is $1/k$.

Results

The nitro analogues of citrate and isocitrate were competitive inhibitors of aconitase regardless of the substrate used, and in no case was a better fit obtained to eq 2 than to eq 1. The K_i values at pH 8 with citrate, isocitrate, or aconitate as the substrate were 39 ± 5, 59 ± 7, and 31 ± 3 μM for the citrate analogue and 1.2 ± 0.2, 1.4 ± 0.2, and 1.03 ± 0.07 μM for the isocitrate analogue.

Assays in the presence of the isocitrate analogue gave nonlinear time courses (Figure 1). When assays were initiated with enzyme, an apparent burst was observed, while, if substrate was added after preincubating enzyme and inhibitor, the assays showed a lag. The final steady-state rate in both cases was the same, indicating the time-dependent inhibition by the isocitrate analogue to be a reversible process. Since the initial as well as the final rate is decreased by the inhibitor, the initial formation of an enzyme–inhibitor complex appears to be followed by a slow conformation change which increases the affinity of the enzyme for inhibitor.

The pK values for the nitro analogues of citrate and isocitrate were determined by spectrophotometric titration at 240 nm to be 10.80 and 9.46, respectively. These titrations were conducted by adding an aliquot of the analogue to a buffer solution at the desired pH and recording A_{240} after sufficient time for equilibration. The approach to equilibrium had a half-time of ~10 min at the pK of the carbon acids, which decreased to less than 1 min 3 pH units above or below the pK. In all inhibition studies the nitro analogues were preequilibrated at the desired pH before use. To determine the final steady-state inhibition of the isocitrate analogue, we preincubated the inhibitor and enzyme until equilibrium was established (30 min for concentrations above K_i and up to 2 h for lower levels). Assays were then initiated with substrate, and the final steady-state rate was determined after completion of the lag. Only the pH range over which aconitase would survive without loss of activity during preincubation (pH 5–8.5) was used in these experiments. In all experiments the inhibitor concentration exceeded that of enzyme [determined by the method of Morrison (1969)] by at least a factor of 5. The pH profiles for inhibition of aconitase by the nitro analogues

are shown in Figure 2. Clearly, both the ionized and undissociated carbon acid forms are inhibitors of aconitase. Values for the K_i of the carbanion (K_{iH}) and protonated (K_{iL}) forms are as follows: nitro analogue of citrate, K_{iL} = 1.7 ± 0.2 mM and K_{iH} = 59 ± 6 nM; nitro analogue of isocitrate (from initial velocities), K_{iL} = 58 ± 4 μM and K_{iH} = 72 ± 5 nM; nitro analogue of isocitrate (from final steady-state velocities), K_{iL}* = 684 ± 32 nM and K_{iH}* = 682 ± 32 pM.

To obtain estimates of the reverse isomerization rate for the complex of aconitase with the isocitrate analogue, we equilibrated enzyme (<0.36 μM) with excess inhibitor (1 μM) in 10 μL and subsequently added it to a 3-mL assay (525 μM isocitrate and 0.1 M Tris, pH 8) so that the final concentration of inhibitor was well below its inhibition constant. The k for the lag under these conditions was 0.064 ± 0.010 min^{-1}.

Discussion

Although Villafranca (1974) has reported that molecules similar to citrate were competitive vs. citrate and noncompetitive vs. aconitate, the nitro analogues of citrate and isocitrate are competitive against all three substrates. In addition, the isocitrate, but not the citrate, analogue shows slow binding behavior similar to that shown by methotrexate vs. dihydrofolate reductase (Williams et al., 1979). The slow increase in inhibition is reversible, since (a) a final steady-state level of enzymic activity is achieved, (b) preincubation of aconitase with the inhibitor for extended periods of time results in an equilibrium beyond which no further effect is seen, (c) after preincubation of enzyme and inhibitor, assays initiated with substrate show an increase in activity with time, finally achieving the same steady-state levels attained with enzyme-initiated assays and (d) analysis of the final steady-state levels of inhibition reveals competitive inhibition.

This type of inhibition can be interpreted in terms of mechanism 5

$$EI^* \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI \underset{k_2}{\overset{k_1 I}{\rightleftharpoons}} E \overset{K_a}{\rightleftharpoons} EA \longrightarrow E + \text{products} \qquad (5)$$

where k_3 and k_4 are much smaller than k_1, k_2, or the rate constants involved in the catalytic reaction. If the level of I is at least 5 times that of the enzyme (as in the present studies), the K_i from initial velocities is k_2/k_1, while the value from final steady-state velocities is

$$K_i^* = (k_2/k_1)k_4/(k_3 + k_4) \qquad (6)$$

so that the equilibrium constant for conversion of EI to EI* is given by

$$k_3/k_4 = K_i/K_i^* - 1 \qquad (7)$$

When this calculation is carried out with K_i values for the carbanion form, k_3/k_4 = 105, while for the protonated form, k_3/k_4 = 83. Thus, both the carbanion and protonated forms of the nitro analogue of isocitrate induce the same conformation change after initial binding and to nearly the same extent.

In mechanism 5, the apparent k for a burst or lag in the time course of the assay when reaction is started with enzyme or, after preincubation of enzyme and inhibitor, with substrate is

$$k = k_4 + k_3(I/K_i)/(1 + I/K_i + A/K_a) \qquad (8)$$

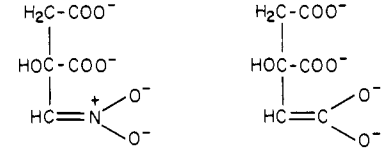
In the experiment where enzyme and inhibitor were preincubated in small volume and then diluted upon addition to an assay mixture to the point where the isocitrate analogue was below its K_i, the second term in eq 8 is very small (about 2% of the first one), so a good estimate of k_4 of 0.063 ± 0.010 min^{-1} can be obtained. Since these experiments were carried out at pH 8 where the carbanion is the inhibitory form, we can use the k_3/k_4 value of 105 to calculate k_3 as 6.6 ± 1.8 min^{-1}. The values of k_3 and k_4 can now be used to calculate expected k values from eq 8 for each of the experiments shown in Figure 1. Table I shows a comparison of calculated and observed k values in these experiments. The agreement is reasonable in view of the difficulty of measuring the rate constants for lags and bursts. (Any drop in rate due to enzyme instability, exhaustion of substrate, buildup of product inhibition, or other causes distorts the results.)

Table I: Comparison of Calculated and Experimentally Determined Values of k for Assay Progress Curves with the Nitro Analogue of Isocitrate Present

nitro analogue of isocitrate (μM)	calcd k (min^{-1})[a]	obsd k (min^{-1})[b]	
		lag	burst
0.5	0.26 ± 0.10	0.27 ± 0.03	
1.0	0.45 ± 0.19	0.50 ± 0.04	
2.0	0.79 ± 0.35	0.62 ± 0.10	0.37 ± 0.01
5.0	1.6 ± 0.74	0.80 ± 0.50	0.70 ± 0.02
10.0	2.6 ± 1.2		1.3 ± 0.05
20.0	3.7 ± 1.7		2.3 ± 0.1

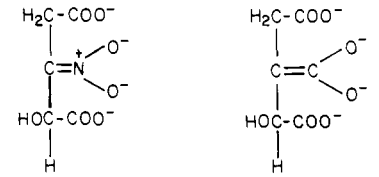
[a] Values were calculated by using eq 8 and K_i = 1.39 ± 0.16 μM, A = 525 μM, K_a = 49.1 ± 4.2 μM, k_4 = 0.063 ± 0.010 min^{-1}, and k_3 = 6.6 ± 1.8 min^{-1}. [b] Data shown in Figure 1 were fitted to eq 4 (see Experimental Procedures). The enzyme concentration used in these experiments was <0.1 μM as determined by titration of the enzyme with the isocitrate analogue (a mixture of stereoisomers) by the method of Morrison (1969). "Lag" refers to experiments where enzyme and inhibitor were preincubated and reaction was started with substrate, while "burst" refers to experiments where reaction was begun by adding enzyme.

In the carbanion form, the nitro analogues of citrate and isocitrate are potent inhibitors of aconitase. Not only does the affinity of the analogues increase 30 000- and 1000-fold upon ionization, respectively, but the Michaelis constants for citrate (157 ± 19 μM) and isocitrate (49 ± 4 μM) are 2700 and 700 (72 000 after the slow isomerization) times greater than K_i values for the carbanion forms of the inhibitors. Tighter binding of an inhibitor than the substrate can be interpreted to indicate that the inhibitor resembles a reaction intermediate or the transition state (Wolfenden, 1976, 1977; Lindquist, 1975). The carbanion forms of the nitro analogues can thus be considered analogues of carbanions formed from citrate and isocitrate during the normal reaction. These carbanions presumably exist as aci-acids:



carbanion of citrate analogue

carbanion of citrate



carbanion of isocitrate analogue

carbanion of isocitrate

Thus, the enzyme preferentially adsorbs molecules with one carbon trigonal and the neighboring one tetrahedral and generates the structures shown above by deforming C-3 of

isocitrate or C-2 of citrate to near planarity (and perhaps also by deforming bond angles from 109 to 120°) so that a proton is readily transferred to a base on the enzyme [where it is not exchanged with solvent while substrates are present on the enzyme (Rose & O'Connell, 1967)], or by twisting and bending aconitate at the appropriate carbon to induce reaction with water (presumably assisted by proton transfer to a base on the enzyme). A similar mechanism has been deduced for fumarase from isotope effect studies by J. S. Blanchard and W. W. Cleland (unpublished experiments) and from inhibition by the carbanion of 3-nitro-2-hydroxypropionate as a transition-state analogue by Porter & Bright (1980). It thus appears that these enzymes use geometric deformation, accompanied by suitable acid–base chemistry, to accomplish catalysis.

It is interesting to note that the protonated forms of the nitro analogues which have only the two carboxyl groups ionized still bind as well or only 10 times weaker than the corresponding substrates. Thus, the nitro group, which has no net charge (although the oxygens have partial negative charge and the nitrogen has positive charge), provides the same or nearly the same binding strength as an ionized carboxyl group. The same is probably true for a protonated carboxyl group, since the V/K values for the substrates and the pK_i for tricarballylate do not decrease at the pH where one of the carboxyls of these molecules becomes protonated (J. V. Schloss, unpublished experiments). The failure of the pK_i value for the nitro analogue of citrate to decrease at pH 4.5, in fact, suggests that one of its carboxyl groups may also be protonated without decreased binding. It appears that both of the carboxyl groups which become aci-acids in reaction intermediates may be bound not to lysines or arginines but by hydrogen bonds to formally neutral groups on the enzyme.

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Chicken Reticulocyte Nuclear Antigen: Its Identification and Relation to Transcriptive Activity in Erythropoietic Cells†

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ABSTRACT: Antibodies to chicken reticulocyte dehistonized chromatin were produced. These antibodies can distinguish between chromatin from reticulocytes and erythrocytes and have no activity with chicken liver chromatin. The antibodies will also differentiate between reticulocytes and erythrocytes by the horseradish peroxidase bridge localization technique. The nonhistone protein components of the antigenic complex are very tightly bound to the DNA, removable by extraction with sodium dodecyl sulfate but not 2.5 M NaCl–5 M urea. Our data indicate that the antigen complexes could actually be present in both reticulocytes and erythrocytes but that chromatin condensation causes a structural masking of the complexes which coincides with the known decline in transcriptive activity of the erythrocyte.

Avian species, in contrast to mammalian species, retain their cell nuclei in erythrocytes through all stages of developmental and cellular differentiation. In the reticulocyte the nucleus is actively involved in transcription of globin and other messenger RNA. The erythrocyte nucleus in comparison is pycnotic and shows very little, if any, transcriptive activity (Barrett et al., 1974).

It has been proposed that nonhistone chromosomal proteins are involved in the regulation of transcription and the differentiation of cells during development (Baserga, 1974; Stein et al., 1974; Jeter & Cameron, 1974; Chiu & Hnilica, 1977). Specifically, we and others have shown that certain nonhistone chromosomal protein–DNA complexes have antigenic activity(s) and that the antibodies induced by the nonhistone–DNA complexes are able to distinguish between the tissue of origin and other tissues including tumor tissue (Chytil & Spelsberg,

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