Aconitase: Its Source of Catalytic Protons†

Donald J. Kuo and Irwin A. Rose*
Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111
Received May 14, 1987; Revised Manuscript Received July 9, 1987

ABSTRACT: An ordinary isotope partition experiment was performed to determine the rate of dissociation of the proton from the donor site for the hydration of *cis*-aconitate. Aconitase in [^{3}H]water was efficiently diluted into well-mixed solutions of *cis*-aconitate. Citrate and isocitrate that were formed within 2 s were more heavily labeled than could be explained by consideration of an isotope effect in the processing of one proton per enzyme equivalent. Control experiments indicate that mixing was much more rapid than catalytic turnover, ruling out incompletely diluted [^{3}H]water as a significant isotope source. Therefore, it appears that significantly more than one enzyme-bound tritium atom (protons) must have been used in the course of the multiple turnover of the enzyme after the dilution was complete. Isotope incorporation reached values in excess of four proton equivalents as a limit with simple Michaelis dependence on *cis*-aconitate. From the half-saturation concentration value for trapping, 0.15 mM, the $t_{1/2}$ for exchange of each of these protons with solvent appears to be ~0.1 s at 0 °C. The large number of protons trapped seems to suggest the existence of a structurally stabilized pool of protons, or water, that communicates between the active site base and the medium in the hydration of *cis*-aconitate. The proton abstracted in the dehydration of [^{3}H]citrate is transferred directly to undissociated *cis*-aconitate to form isocitrate without dilution, or *cis*-aconitate having dissociated, the tritium passes to the medium, presumably through the pool of bound protons indicated above. *All* of the citrate-derived protons can be found in isocitrate if *cis*-aconitate is added in sufficient concentration. Therefore, the abstracted proton dissociates slowly, if at all, from the enzyme in all intermediates except those from which *cis*-aconitate has dissociated. Half of the citrate-derived proton is trapped by ~1 mM *cis*-aconitate with a Michaelian dependence. Citrate, as well as *trans*-aconitate and tricarballylate, competes with *cis*-aconitate in its utilization of the citrate-derived proton. Unlike *cis*-aconitate, these acids form complexes from which the citrate-derived proton can dissociate, and in the case of citrate, a functional complex results. The rate and mechanism of proton dissociation from E–H$^+$·citrate are unknown.

The abstraction of a substrate-bound proton is a fundamental process that occurs in many examples of enzyme catalysis (Rose, 1970). Transfer to and from carbon in hydratases and transfer to and from oxygen in kinases and dehydrogenase are a few examples. In many cases the abstracted proton can be found in a stable position of the departing product, which provides the best evidence for general acid/base catalysis by enzymes. In cases in which water must be the ultimate proton donor or acceptor, it is often assumed that the enzyme intervenes as the immediate agent of the chemical process (Cleland, 1982; Viola & Cleland, 1978). Evidence for enzyme acting as the base in a dehydration reaction exists for aconitase because the proton that is abstracted in the dehydration of citrate can be found either in water when *cis*-aconitate is produced or in the alternate product, isocitrate (Rose & O'Connell, 1967). When proton abstraction is not balanced by transfer to product as when free *cis*-aconitate is formed from citrate and the enzyme acts in the proton abstraction, there must be an additional reaction at the active site, proton transfer to solvent. Little is known about this process: Is it a rapid process or possibly rate determining for the catalytic cycle? Does, in fact, the next round of a dehydration reaction require that the previously abstracted proton leave before the substrate will interact? Is the proton that is transferred to the water the same one that comes from the substrate in each cycle, or is a proton relay part of the reaction process? The present study provides data relevant to these questions for the aconitase reaction.

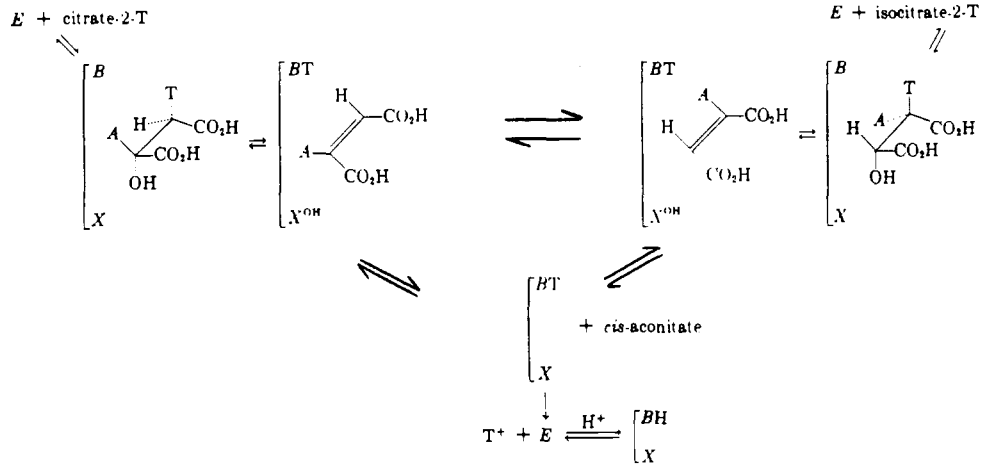
† This work was supported by National Institutes of Health Grants GM-20940, CA-06927, and RR-05539 and by an appropriation from the Commonwealth of Pennsylvania.

The mechanism of the citrate–isocitrate isomerization catalyzed by aconitase is shown to be one of successive dehydration and hydration by the observation that free *cis*-aconitate is a reaction side product (Glusker, 1971). The unusual ease of dissociation of this intermediate of catalysis, at least equal to the rate of isocitrate formation, may be rationalized from the stereochemistry of the citrate to isocitrate conversion and the observation of intramolecule tritium transfer, making it appear that there must be reorientation of the plane of the *cis*-aconitate intermediate with respect to the abstracted proton, T, in each catalytic cycle, Scheme I. Preservation of this proton for transfer without dilution was found in the citrate formed at early times from [3-^{3}H]isocitrate (Rose & O'Connell, 1967), indicating either that the reactive basic group is not an amine or if it is that it does not undergo positional isotope exchange in its protonated form. Further evidence for the role of enzyme as the reacting base comes as the best explanation for significant intermolecular transfer catalyzed by the enzyme: [3-^{3}H]-2-methylisocitrate + *cis*-aconitate → 2-methyl-*cis*-aconitate + [3-^{3}H]isocitrate (Rose & O'Connell, 1967).

More recently, active aconitase of muscle has been characterized as an iron–sulfur protein, containing a single Fe_4S_4 cubic cluster per 80-kDa enzyme (Kent et al., 1982; Kennedy et al., 1983; Ryden et al., 1984). Electron paramagnetic resonance (EPR) and Mössbauer effect studies appear to support the view that a unique ferrous atom of the cluster, X in Scheme I, is directly linked to the –OH of citrate in the course of dehydration (Emptage et al., 1983). The inability to show hydroxide transfer in the conversion of [*hydroxy*-^{18}O]citrate to isocitrate (Rose & O'Connell, 1967) or indeed in the reaction in which [*hydroxy*-^{18}O]methylisocitrate +

0006-2960/87/0426-7589$01.50/0

Scheme I



cis-aconitate → isocitrate (unpublished, Rose & O'Connell, 1973) indicates that the substrate-derived –OH group is much more readily exchanged with water within the intermediate complex than is the abstracted proton.

The initial goal of the present study was to determine the rate of dissociation of the abstracted proton, by determining how much *cis*-aconitate was required to suppress its loss. In addition to generating tritium-labeled E–H^+ from labeled citrate, we have used a variation of the isotope-trapping method (Rose et al., 1974) in which the enzyme is incubated briefly with [^{3}H]water and the ability of *cis*-aconitate to compete for the enzyme-bound tritium is determined.

Theory

In the general isotope-trapping method, enzyme and labeled substrate S_1* are incubated briefly to form a presumed functional Michaelis complex. The labeled complex will be converted to product if, following the addition of a solution of the second substrate S_2 and unlabeled S_1, the catalytic process is more rapid than the dissociation of S_1* and if S_1* does not dissociate from the ternary complex, E·S_1*·S_2. In the latter case a surprisingly simple equation describes the substrate dissociation rate constant k_{off} and the concentration of S_2 required for half-maximal trapping K_{tr} in terms of the steady-state parameters k_{cat} and K_m of S_2 (Rose et al., 1974):

$$k_{off}/k_{cat} = K_{tr}/K_m \quad (1)$$

The dissociation constant of the active site binary complex should be obtained by determining how the amount of labeled product varies with the concentration of S_1* in the primary incubation, or pulse, at constant S_2 in the chase. The minimum number of active sites per enzyme should be given by the largest amount of substrate that can be trapped after correction for the isotope that is incorporated from the S_1 + S_1* mixture. A nonfunctional analogue of the trapping substrate will increase K_{tr} as might be expected for a competitive inhibitor only if the E·S_1*·inhibitor complex is able to exchange S_1* for S_1. An analogue of S_2 that does not allow exchange of S_1* to occur in the complex should have no effect on the concentration of S_2 required to achieve half-maximal trapping. Such an inert inhibitor would only increase the time required to obtain the same partition of isotope.

Materials and Methods

Enzyme. Two preparations of aconitase were made from rat heart mitochondrial extracts (Crane et al., 1956) which were directly treated with cellulose CM-52 (Villafranca & Mildvan, 1971). The enzyme was eluted with *cis*-aconitate, and the eluate was precipitated by ammonium sulfate fractionation (55–70%). The dialyzed enzyme was chromatographed on a Blue Dextran–Sephadex column and eluted with tricarballylate according to Scholze (1983). Active fractions were concentrated and diluted successively to remove the tricarballylate on an Aminco concentrator and ultrafiltrator in a medium of 25 mM triethanolamine–acetate, pH 8.0, and 1 mM dithiothreitol (DTT) in which the enzyme was stable at −70 °C.

Aconitase was activated before each experiment as follows: equal volumes of aconitase and activating solution [100 mM 1,4-piperazinediethanesulfonic acid (PIPES), 30 mM cysteine, and 15 mM $Fe(NH_4)_2(SO_4)_2$ at pH 7.0] were mixed under N_2 at ambient temperature. After 40 s the activated enzyme, put on ice, was stable for at least 10 min.

Enzyme activity was determined by coupling to isocitrate dehydrogenase at 25 °C: citrate (10 mM), triethanolamine–acetate buffer (100 mM, pH 8.0), $MgCl_2$ (10 mM), $NADP^+$ (1 mM), isocitrate dehydrogenase (5 units), and <50 milliunits of aconitase. Absorbance at 340 nm increased linearly without a lag at a constant rate for several minutes. The activated rates of the two preparations, protein-bound iron, and protein assays are recorded in Table II. Both preparations were less active than expected from protein determinations, 58 and 66% pure. On the basis of iron determination and the expectation of one Fe_3S_4 group per enzyme before activation, the preparations were 78 and 97% of their expected activities, respectively.

Iron content of the unactivated preparation was determined according to Beinert (1978) to determine the upper limit of aconitase present. Samples were assayed directly (free iron) or after being heated in H_2SO_4 and HNO_3 to dryness in a Pyrex tube. Ferrozine (1.4 mM), sodium ascorbate (80 mM), and sodium acetate (120 mM, pH 4.8) were added, and the absorbance was read at 560 nm. From a standard curve with $Fe(NH_4)_2(SO_4)_2$, ϵ^{560} = 24.3 mM^{-1}. With enzyme activity as the measure of protein, assuming 12 units/mg for all the aconitase present, the Fe/aconitase ratios for the two preparations were 3.8 and 3.0 to 1 (Table II).

Chemicals. Isocitrate dehydrogenase isolated from porcine heart, type VI, was purchased from Sigma Chemical Co., and glutamate dehydrogenase isolated from beef heart was purchased from Boehringer Biochemicals. Yeast hexokinase (Boehringer) was treated with trypsin to increase its affinity for glucose (Rose et al., 1974). Yeast enolase from Sigma was used without further purification. Fluorocitrate was converted from the barium salt (Sigma), and *cis*-aconitic acid anhydride (Sigma) was hydrolyzed to a neutral solution, which was stored at −70 °C.

Table I: Aconitase-Bound ^3H Trapped by *cis*-Aconitate[a]

pulse						
aconitase, nmol	0.42	0.49	0.44	0.44	0.41	0.42
[^{3}H]water, cpm/nanoatom of H	5760	7980	←	5673	→	8700
chase						
cis-aconitate, mM	5	1	0.5	0.2	0.05	0.1
^3H in citrate + isocitrate						
exptl, cpm	13280	15540	9720	9100	4480	7060
control, cpm	1520	1284	1370	1380	1230	620
difference, nmol	2.03	2.16	1.45	1.37	0.57	0.90
^3H fixed/aconitase	4.8	4.4	3.3	3.1	1.4	2.1

[a] Aconitase (0.32 mM based on activity) was diluted into activating solution containing [^{3}H]water for 40 s and then put on ice; 5 μL of this *pulse* solution containing ~0.4 nmol of aconitase was added to 5 mL of stirred solution (on ice) containing *cis*-aconitate (0.05–1 mM) or to 1 mL of 5 mM *cis*-aconitate and PIPES (10 mM, pH 7.0) with bovine serum albumin (2 mg/mL). Trichloroacetic acid to 0.2 M was added at 2 s. Each experiment had a control in which *cis*-aconitate was added to the diluted pulse. Citrate and isocitrate were isolated together. Their combined counts, corrected for the control, divided by half the specific activity of the [^{3}H]water, give the atoms of ^3H of the pulse solution that were trapped (difference row in table) from which the number of protein-bound tritium equivalents per equivalent of enzyme is calculated. From the enzyme used, the equivalents of ^3H fixed in citrate and isocitrate/equivalent of enzyme were calculated.

Labeled Substrates. ^3H_2O (10 Ci/mL, Amersham) was used directly. [2-^{3}H]Citrate was prepared by conversion of *cis*-aconitate to the equilibrium of aconitase products, citrate/isocitrate/*cis*-aconitate = 88/8/4 (Thomson et al., 1966). The incubation was quenched with trichloroacetic acid and chromatographed through Dowex 1-Cl$^-$. [3-^{3}H]Isocitrate was removed as L-glutamate with isocitrate dehydrogenase and glutamate dehydrogenase followed by chromatography on Dowex 1-formate. The column was eluted with a linear gradient of water and 6 N formic acid, and [2-^{3}H]citrate was recovered with a specific activity of 4.24×10^4 cpm/nmol.

Isolation of Isocitrate and Citrate Formed in [^{3}H]Water (Table I). Trichloroacetic acid insoluble material was discarded after centrifugation, 5 μmol each of citrate and isocitrate was added, and the trichloroacetic acid was removed by extraction with ether (4 × 10 mL). [^{3}H]Water was removed by passage through a 1-mL column of DE-52 cellulose washed well with water and counted. The acids were recovered (99%) by elution with 1 N HCl, lyophilized, and applied to a HPLC column of Bio-Sil (ODS-5S 250 × 4 mm) and eluted with a linear gradient beginning with $(Bu)_4N{\cdot}HSO_4$ (10 mM)/4-morpholineethanesulfonic acid (MES, 50 mM, pH 5.5) and mixing with acetonitrile (0–10% in 20 min). Citrate and isocitrate peaked together at 13–14 min, liquid scintillation counting being used to follow the elution. All counting was done in 5 mL of scintillation fluid (Optifluor, product of Packard Instrument Co.) with 1 mL of sample. Counting efficiency for all samples including [^{3}H]water was found to be the same.

Characterization of ^{3}H-Labeled Products. Mixtures of presumed [^{3}H]citrate and [^{3}H]isocitrate were converted to L-glutamate by isocitrate dehydrogenase and glutamate dehydrogenase with or without aconitase, and the resulting solution was chromatographed on Dowex 1-acetate. [^{3}H]Water was in the breakthrough and column wash, and [^{3}H]glutamate was recovered by elution with 0.5 M acetic acid. Counts found as glutamate come entirely from [3-^{3}H]isocitrate when aconitase is absent. Additional radioactivity in glutamate and in water with aconitase present resulted from [2-^{3}H]citrate, ~51% of the total. These and the glutamate counts without aconitase make up all of the label fixed in the experiments of Figure 1.

Mixing Procedures. Several procedures for mixing were tested and found to give similar results: for the data of Table I the cold chase solution (1 or 5 mL in a 20-mL glass counting vial flushed with N_2) was stirred at ~2500 rpm with a 12-mm magnetic bar. The 5 μL of aconitase, activated in [^{3}H]water, was added by forced injection from a Pipetman. Essentially identical results were obtained with slower mixing rates or if the chase solution was added to the enzyme in a glass 12-mL conical centrifuge tube stirred magnetically.

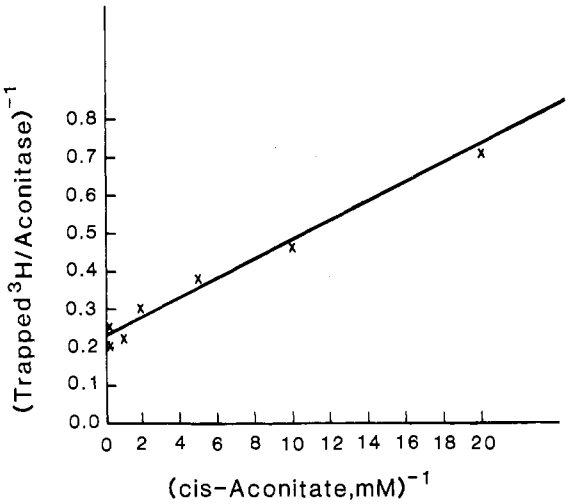


FIGURE 1: Trapping by *cis*-aconitate of E–H$^+$ derived from [^{3}H]water. Data from Table I and includes all additional experiments of this kind.

RESULTS

EH$^+$ Derived from Water. (A) Effect of [cis-Aconitate] in Preventing the Dissociation of Tritium Derived from Water. Aconitase was freshly acitvated in [^{3}H]water, the "pulse", and diluted (200–1000-fold) into a rapidly stirred ice-cold solution containing *cis*-aconitate in the concentration range of 0.05–5 mM, the "chase" solution, Table I and Figure 1. The reaction was done at ~0 °C to avoid excessive incorporation of isotope from the medium. The rate of *cis*-aconitate disappearance was ~6 s^{-1} under these conditions. The reaction was quenched with acid (0.2 N final), at 2 s. Citrate and isocitrate (5 μmol each) were added, and the two were recovered together as described (Materials and Methods) and counted. The unexpected result of this experiment is that amounts of tritium were trapped in great excess of one per equivalent of protein, Table I. Between 1.4 and 4.8 enzyme equivalents of tritium were fixed when *cis*-aconitate was varied from 50 μM to 5 mM. Similar results were obtained with a second enzyme preparation. To calculate the amount of enzyme present in each incubation, the activity under standard conditions was determined and compared with the highest activity reported for this enzyme, 12 units/mg (Kennedy et al., 1983).

A significant amount of radioactivity was found in the two kinds of controls in which the enzyme in [^{3}H]water was added either to acid-containing H_2O/*cis*-aconitate or to ice-cold chase solution lacking the *cis*-aconitate to which the *cis*-aconitate

Table II: Comparison of Aconitase Activity and Protein and Iron Content

preparation	1	2
activated rate, units/mL	310	754
protein from activity, mM[a]	0.32	0.78
protein from Lowry, mM[b]	0.58	1.18
protein from Fe/3.0, mM[c]	0.41 ± 0.06 (7)	0.80 ± 0.07 (16)

[a] Based on rate assumed for fully active enzyme, 12 units/mg and molecular mass of 80 kDa. [b] Lowry et al. (1951), using bovine serum albumin as a standard. [c] Assuming the only source of Fe is unactivated aconitase (3 Fe/mol) in 7 and 16 determinations.

was added for 2 s before the addition of acid. The purpose of these two controls was to determine the counts that would be incorporated due to the presence of substrate that might contaminate the enzyme and as a consequence of the conversion of *cis*-aconitate to citrate plus isocitrate at the fully diluted specific activity of the water in the 2-s chase. As may be calculated from Table I, these controls represent from ~0.1–0.25 of an enzyme equivalent. They are believed to arise from substrate that contaminates the enzyme preparation for the following reasons: The two kinds of controls were quite similar in magnitude. The incorporation that might have been expected in the diluted control would be negligible due to the slow rate of the enzyme at 0 °C. Thus, $\sim 6\ s^{-1} \times 2\ s = 12$ equivalents in 1000-fold diluted [^{3}H]water should represent only ~0.012 equivalents of tritium at the initial specific activity. The counts incorporated in the controls are stereospecifically placed in the products since they are removed by incubation with aconitase and dependent on the presence of enzyme in the experiment. Indeed, difficulty in removing substrates from aconitase with an apparent binding constant of ~2 μM has been noted by Schloss et al. (1984).

(*B*) *Iron Content of Aconitase Preparation.* Since the content of aconitase in Table I is based on the assay of enzyme activity, the presence of active enzyme with lower effectiveness in the standard assay would cause a falsely low estimate of active sites leading to an exaggerated stoichiometry. To quantitate the maximum aconitase possible independent of either catalytic activity or protein determination, it was desirable to evaluate total iron content of the preparation. On the basis of Fe_3S_4 per one equivalent of unactivated enzyme (Beinert et al., 1983; Kennedy et al., 1983), this places an upper limit on the amount of active enzyme. As shown in Table II, the Fe content is consistent with the use of the rate of the activated enzyme as the basis for calculating aconitase concentration as done above.

It should be clear at this point that the incorporation of tritium in excess of the controls is assumed to require multiple turnovers of the enzyme when H^+ with the high specific activity of the [^{3}H]water of the preincubation is used. The isolated labeled products are indeed citrate and isocitrate (Materials and Methods). The elution of counts shows a peak coincident with the authentic acids. The tritium is fully exchange with water when activated aconitase is added, and about half of the tritium of the peak can be converted to glutamate by treatment with a combination of isocitrate dehydrogenase and glutamate dehydrogenase (not shown).

The stereochemical requirements of the aconitase reaction as they are known (Glusker, 1971) allow only one C–H position of each product to be labeled from tritiated water in its formation from *cis*-aconitate. This was confirmed with this enzyme preparation by showing that in a prolonged incubation no more than one ^{3}H equivalent was incorporated into the combined acids.

An inverse isotope effect should not be responsible for the high tritium content of the aconitase products: Equilibrium isotope fractionation that would enrich E–H^+ in ^{3}H relative to water would require an unusual protein residue. In the range of possibilities the enrichment values expected are 0.37, ~1, and ~1 for –SH, –NH and $-CO_2H$ groups (Schowen, 1972). A kinetic isotope effect *favoring* tritium incorporation is not expected from any studies of the enzyme: Only small normal isotope effects have been found with substrates (Thomson et al., 1966). In a multiple turnover experiment done in [^{3}H]water and *cis*-aconitate at very high concentration (300 mM), the averaged specific activity of citrate and isocitrate formed when the reaction was far from equilibrium was close to that of the water (Rose, 1977). Failure to observe net discrimination against the heavy atom was believed to result from commitment of E–H^+ to reaction when *cis*-aconitate is above its K_m. This explanation would suggest that 4–4.8 ^{3}H atoms represent *all* of the atoms present in a proton donor pool in the active site.

Table III: Simulation of [^{3}H]Water/Aconitase/*cis*-Aconitate + H_2O by [^{3}H]Glucose/Hexokinase/ATP + Glucose

glucose (mM)	[^{3}H]glucose trapped	
	cpm	nmol/nmol of E[b]
1	8350	0.96
20	8028	0.97
200	7616	0.83

[a] An ice-cold solution (4.5 μL) of yeast hexokinase (0.2 nmol), [6-^{3}H]glucose (1 mM, $\sim 4 \times 10^4$ cpm/nmol), and $MgCl_2$ (10 mM) in 0.1 M KP_i, pH 7.0, was injected into 5 mL of a cold stirred chase solution, alike in buffer and $MgCl_2$ and including ATP (5 mM) and D-glucose as noted. Radioactivity in glucose 6-phosphate generated within 2 s was determined. As a control, ATP was used to initiate parallel incubations after the dilution to 5 mL was made. [b] Corrected for [^{3}H]-glucose 6-phosphate formed in the prediluted controls (9.1, 8.2, and 19.2% at 1, 20, and 200 mM glucose, respectively).

(*C*) *Mixing Artifact.* Applying the tracer partition method to aconitase with tritiated water as the source of labeled substrate and *cis*-aconitate in unlabeled water in the chase solution is directly comparable to an earlier experiment (Rose et al., 1974) with labeled glucose and hexokinase using ATP and unlabeled glucose in the chase solution with the possibly important difference that the glucose in the chase was made 500-fold more concentrated that that used to load the enzyme. In the experiment of aconitase in [^{3}H]water, the *concentration* of water in the solution that contains the *cis*-aconitate cannot be made to exceed that of the water of the pulse solution. Thus, as the enzyme encounters *cis*-aconitate, the tritium not bound to the enzyme is diluted in proportion to the mixing of the two solutions whereas the *cis*-aconitate increases abruptly to above its K_m of 13 μM in the earliest phases of mixing, possibly initiating the reaction before dilution is final or perhaps even significant. This possibility, termed "the mixing artifact", has been discussed as a possible limitation of the isotope-trapping method, especially when applied to enzymes of rapid turnover (Rose, 1980). It is characterized by excessive trapping that may depend on the efficiency of mixing and by a failure to observe saturation kinetics.

To evaluate the contribution of reaction that occurs before extensive dilution of the [^{3}H]water, the isotope-trapping experiment reported earlier (Rose et al., 1974) with labeled glucose and yeast hexokinase was repeated with the glucose (1 mM) in the chase solution equal to that with which the hexokinase had equilibrated, Table III. The mixing condition were the same as used in Figure 1 with aconitase. The hexokinase used had about twice the turnover rate in situ, 13 s^{-1} at 0 °C, as that of the aconitase in its experiment. The ATP concentration at 5 mM was in great excess of K_{tr}, ~0.18 mM, so that any glucose that becomes associated with the enzyme

Table IV: Tritium Transfer of [2-^{3}H]Citrate to [3-^{3}H]Isocitrate at Equilibrium Concentration of *cis*-Aconitate–Citrate (1:30)[a]

	citrate (mM)				
	0.1	7	10	50	100
extent of reaction (%)	3.9	11.9	12.2	2.7	3.3
percent as isocitrate	18.3	17.5	18.7	20.7	19.2

[a] The incubation at 25 °C contained in 50 μL PIPES buffer (0.1 M, pH 7.0), NADP (1 mM), $MgCl_2$ (1 mM), ADP (1 mM), serum albumin (50 μg), ammonium acetate (50 mM), glutamate dehydrogenase (2 units), isocitrate dehydrogenase (1 unit), [^{3}H]citrate (8×10^5 cpm, in the concentration cited), *cis*-aconitate (at 0.033 of the citrate), and aconitase at milliunit with citrate at 0.1 and 7 mM and 17 milliunits at other concentrations. Incubation times varied from 1 to 40 min to achieve the noted extent of [^{3}H]citrate utilization. Fluorocitrate was added (5 mM) to stop the aconitase and the incubation continued for 10 min more to allow the processing of any remaining isocitrate to be complete. Protein was precipitated with acid and the supernatant analyzed on Dowex 1-acetate for tritium in water and glutamate, the sum of which gives extent of reaction.

after the first catalytic cycle with bound radioactive glucose would also be converted to glucose 6-phosphate. The amount of glucose trapped at the initial specific activity was close to the one equivalent expected (Rose et al., 1974) and was essentially the same with either 1 or 20 mM glucose in the chase. At the very worst, taking the result with 200 mM glucose in the chase to be free of any mixing artifact, the correction for nonideal mixing would only be 15%. From k_{off} of glucose and the binding constant of glucose calculated by isotope trapping (Rose et al., 1974), the rate constant for binary complex formation, E + Glc → E·Glc, should be $\sim 10^6$ M^{-1} s^{-1}. Therefore at 10^{-3} M glucose, the pseudo-first-order rate constant is $\sim 10^3$ s^{-1} for glucose association. This is in competition with the dilution of the isotope that occurs as the reaction mixture is becoming complete with S_2. A 15% error due to real time of mixing would be explained if 1 ms after mixing the glucose that had diffused to the enzyme would have been diluted at least 7-fold giving a mixing rate at least 7×10^3 s^{-1}, which is much greater than the turnover of aconitase at 0 °C, ~ 6 s^{-1}. It follows from this that the tritium incorporated into citrate and isocitrate in Figure 1 must have been bound to the enzyme at the time of mixing and could not have come from incompletely diluted medium as the *cis*-aconitate was added.

In a second evaluation of the adequacy of mixing, enolase and [^{3}H]water were mixed as in the aconitase experiment and chased with phosphoenolpyruvate (PEP) and Mg^{2+} in a 500-fold dilution. The limit of ~ 0.3 enzyme equivalent of ^{3}H at the initial specific activity of water was found in 2-phosphoglycerate. This value was not increased by use of [^{3}H]water in D_2O in the pulse and therefore does not represent a low fractionation factor in the pulse. Whether the low value is due to incomplete loading in the pulse at pH 6.5 and 7.5 or exchange during the chase, the conclusion remains that mixing was rapid relative to a turnover rate of ~ 2 s^{-1} under the chase conditions, 0 °C with PEP at $\sim 10^4$ times its K_m.

E–H$^+$ Derived from Citrate. When [2-^{3}H]citrate is converted entirely to isocitrate in the presence of isocitrate dehydrogenase at 20 °C, about 80% of the ^{3}H is found in water (Rose & O'Connell, 1967). This presumably corresponds to the partition of E–^{3}H·*cis*-aconitate between *cis*-aconitate and [^{3}H]isocitrate. During most of the time of such an experiment the *cis*-aconitate, which is formed initially at 1.8 times the isocitrate rate, is limited by equilibrium to a level that cannot exceed 5% of the citrate. The ability of added *cis*-aconitate to increase the fraction of tritium found in isocitrate to more than ~20% should provide a measure of the trapping constant, K_{tr}, and from this the dissociation rate constant of the tritium derived from citrate. These studies were done at 20 °C, not 0 °C, to be certain that the isocitrate dehydrogenase would prevent accumulation of isocitrate, which would allow it to be detritiated by the aconitase.

In an incubation containing both [2-^{3}H]citrate and *cis*-aconitate, aconitase acts on the *cis*-aconitate preferentially in keeping with its predicted 20–30-fold greater k_{cat}/K_m. To overcome this problem of changing concentrations, the [^{3}H]citrate/*cis*-aconitate ratio was set at equilibrium, and the two substrates were varied together to establish different *cis*-aconitate concentrations. In this case if 20% of the [2-^{3}H]citrate was utilized, the time-average *cis*-aconitate concentration would be within 10% of its initial value. The reaction was followed with $NADP^+$ and isocitrate dehydrogenase. After about 20% conversion of citrate to α-ketoglutarate in the coupled system, glutamic dehydrogenase and NADPH were added to generate [^{3}H]glutamate, which could easily be separated from [^{3}H]citrate and [^{3}H]water on Dowex 1-acetate. Surprisingly, when the trapping experiment was done in this way, Table IV, *cis*-aconitate was unable to alter the partition of citrate-derived tritium at concentrations 20-fold greater than that which trapped half the water-derived tritium. It would appear from this that the only tritium found in isocitrate comes from intramolecular transfer from the E–H·*cis*-aconitate complex that is generated directly from citrate. This result is not only contrary to the published observation (Rose & O'Connell, 1967) of intermolecular transfer of tritium derived from either citrate or 2-methylisocitrate but also implies that the E–H$^+$'s derived from water and citrate are somehow different and not interconvertable. A solution to this dilemma is at hand if E–^{3}H$^+$ derived from citrate were to react with citrate, not in a dead-end reaction but so that the tritium of such a complex were to readily dissociate: E + [^{3}H]citrate → E–^{3}H + *cis*-aconitate; E–^{3}H + citrate → E–^{3}H·citrate → E·citrate. With the net flux toward isocitrate due to the presence of isocitrate dehydrogenase and the interconversion of citrate and *cis*-aconitate being equal at their equilibrium, it would have to be true that citrate causes the exchange of E–^{3}H more readily than *cis*-aconitate reacts with it to form isocitrate. Therefore, to apply the isotope-trapping method to E–H$^+$ derived from citrate, one must use *cis*-aconitate at concentrations that inhibit reaction of citrate and that lead to dilution of the citrate.

A further demonstration of the ability of tricarboxylic acids to facilitate proton dissociation from E–H$^+$ is seen in Table V. An incubation containing initially 0.1 mM [^{3}H]citrate

Table V: Effects of *trans*-Aconitate and Tricarballylate on Tritium Transfer in the Presence of *cis*-Aconitate[a]

	additions						
	none			*trans*-aconitate		tricarballylate	
% reaction[b]	0.22	0.48	1.18	0.28	0.53	0.42	0.72
% tritium transfer[c]	51	44	36	31	28	29	17

[a] Each incubation contained [^{3}H]citrate (0.1 mM, 8×10^5 cpm) and *cis*-aconitate (1.0 mM) supplemented as in Table IV. Further additions as noted were either *trans*-aconitate (20 mM) or tricarballylate (20 mM). Successive early samples were taken and analyzed as before. [b] 100 × [[^{3}H]isocitrate + [^{3}H]water]/[total ^{3}H]. [c] 100 × [[^{3}H]isocitrate]/[[^{3}H]isocitrate + [^{3}H]water].

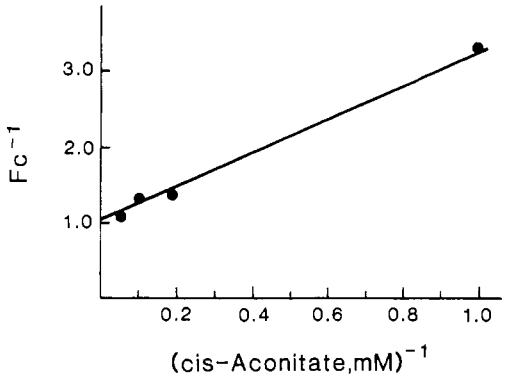


FIGURE 2: Trapping by *cis*-aconitate of E–H$^+$ generated from [^{3}H]citrate. The procedures were as in Table V with 0.1 mM citrate and 0.75–3.0 milliunits of aconitase in going from low to high *cis*-aconitate. Samples were taken to determine the best value for the partition ratio within <1% of the extent of reaction of the [^{3}H]citrate. F_c represents the fraction of the citrate-derived tritium found in isocitrate (glutamate) after correction for intramolecular transfer.

and 1 mM *cis*-aconitate was shown to give about equal [^{3}H]isocitrate and [^{3}H]water if the aconitase reaction was stopped when only 0.22% of the tritium had been utilized. This fell rapidly half-way to the intramolecular limit of ~20% at only 1.2% utilization, reflecting the change in citrate/*cis*-aconitate toward equilibrium. In the presence of the aconitase inhibitors *trans*-aconitate and tricarballylate, not only was the reaction of the [^{3}H]citrate slowed as expected, but at about the same extent reaction much less intermolecular transfer due to *cis*-aconitate was seen. Like citrate they must interact with E–H$^+$ and allow proton dissociation to occur. The concentrations of inhibitors, 20 mM, were much greater than their K_i values: 0.2 mM for *trans*-aconitate (Ramsey et al., 1981) and ~3 mM for tricarballylate (Villafranca, 1974). However, the initial *cis*-aconitate was ~80 fold greater than its measured K_m of 13 μM.

To quantitate the ability of *cis*-aconitate to trap E–H$^+$ that is derived from citrate, it is necessary to restrict the measurements to very limited extents of reaction, not only to avoid the change that would occur in *cis*-aconitate concentration but also to avoid formation of citrate that would further activate the exchange. A constant labeling source, [2-^{3}H]citrate initially at 0.1 mM, and varying *cis*-aconitate, 0.1–10 mM, were used with low levels of aconitase activity compared with that of the dehydrogenases. The aconitase reaction was stopped with fluorocitrate but the incubation continued to be certain that all the [^{3}H]isocitrate has been converted to glutamate. As seen in Figure 2, complete trapping of tritium derived from citrate could be demonstrated with a half-maximum for *cis*-aconitate of 2.2 mM.

Discussion

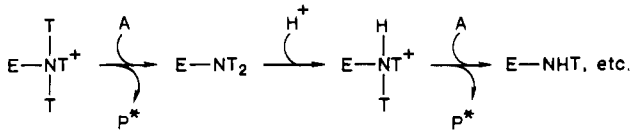
At the outset of this work it was expected that no more than one of the tritium atoms that might exchange into the enzyme during the 40-s exposure to high-activity [^{3}H]water would be trapped in the subsequent reaction with *cis*-aconitate. The precise amount that could be trapped would depend on the pH of the medium and the pK of the active site base. The fact that at least three and possibly more tritiums can be trapped requires us to discard this limited perspective.

On the basis of the extent of ^{3}H transfer and its intramolecular character, Rose and O'Connell (1967) concluded that the carrier of protons between citrate and isocitrate must be a nonprotonic base. Preserving this model, the multiproton pool must be distinct from the acid form of this base although both may become labeled in the ^{3}H pulse. In speculating on the nature of these extra protons we are considering enzyme-bound protons that are not immediately involved in the hydration reaction but which are a more immediate source of these protons than is bulk water. The possibility that the 80-kDa, single polypeptide, aconitase molecule might contain more than one active site is contrary to analysis by EPR of one substrate per Fe_4S_4 center per 80 kDa (Emptage et al., 1983), the presence of one reactive sulfhydryl group measured with phenacyl bromide (Johnson et al., 1977), and the requirement for one additional Fe^{2+} to activate the enzyme (Gawron et al., 1974). The occurrence of one Fe_4S_4 center that performs an active site function is noteworthy because it leads to the proposal that four proton donors might be part of a cubic assembly in which each iron has an identical catalytic role. This contrasts with the current view in which the assembly is monofunctional on the basis of the accessibility to oxidation and facile exchange of only one iron atom without mixing (Kent et al., 1982; Kennedy et al., 1983), although full labeling with ^{59}Fe of all four positions is seen in time (Kennedy et al., 1983). These observations could be explained by the combination of some element of asymmetry in the active site (not surprising for an enzyme) that required a particular iron to be exposed in the absence of substrate. This proposal, which is functionally equivalent to four active sites, provides a rationale for the presence of an iron–sulfur cluster in a nonoxidative role but leaves unaddressed the nature of the catalytic mechanism that would take advantage of such a symmetry of structural elements. Alternative to this one might suggest that only one iron functions in the –OH group transfer but that the structure as a whole provides the basis for holding at least three protons equidistant from the iron. Detailed ENDOR ^{17}O studies of aconitase (Telser et al., 1986) show the simultaneous binding of one water and one nitroisocitrate molecule to the singular Fe atom that seems to abstract the substrate –OH groups of citrate and isocitrate. In this case the free enzyme would have two H_2O molecules on an Fe of expanded valance, or from which a cysteine ligand is replaced by H_2O. These authors suggest that this cysteine might act as the acid/base of proton transfer. This model provides a source of four protons and a base in proximity to them. The source of protons should not have the stability required for at least four *cis*-aconitate molecules to become hydrated however. These speculations only serve to indicate the importance of detailed structural information from crystallographic studies. It will be of interest to know if multiple proton trapping will be found with other Fe_4S_4 hydratases and not those that do not contain the iron/sulfur structure.

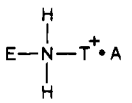
The most extreme extension of the strict acid/base concept of proton transfer that might be considered could allow as many as six enzyme hydrogens to appear in a total of six citrate plus isocitrate molecules. Let it be supposed that a specific lysine ϵ-NH_3^+ residue serves as a proton donor at each face of the *cis*-aconitate plane. *cis*-Aconitate, at high concentration, might trap all of these hydrogens, half in citrate and half in isocitrate, if the only form in which they exchange with the medium is the free enzyme form. Indeed, high *cis*-aconitate was able to capture all of the tritium of E–H$^+$ derived from citrate, Figure 2. This model is not contrary to the data in which [3-^{3}H]isocitrate was converted to citrate without dilution in ^{3}H content (Rose & O'Connell, 1967) as long as tritium, once donated to the enzyme, moved readily between the two active site bases and if isocitrate reacting with E–^{3}H suppressed exchange with the medium. In this mechanism the proton removed from one molecule of isocitrate would always be found on citrate from a subsequently reacting isocitrate. This ex-

planation seems to be ruled out by previous studies (Rose & O'Connell, 1967) that show most of the transfer to be intramolecule. Furthermore, at high concentrations, [2-^{3}H]citrate was not able to alter the partition of ^{3}H between [^{3}H]water and [^{3}H]isocitrate in the absence of added *cis*-aconitate (data not shown) and as shown in Tables IV and V facilitates proton dissociation, making it unlikely that such a process occurred in the reverse direction with isocitrate.

A single base mechanism with $-NH_2$ as base would allow intramolecular, conservative, and multiple transfer. The $-NH_3^+$ group could be thought to undergo several catalytic cycles in the presence of a high amount of *cis*-aconitate so that virtually all of its hydrogens would be recovered in products, P*:



Intramolecular transfer in the citrate → isocitrate does not rule out a proton-containing base if positional exchange due to torsional rotation in the intermediate complex



is slow compared with proton transfer to *cis*-aconitate. Therefore, an $-NH_2$ group that is rapidly protonated and slowly deprotonated in the $-NH_3^+$ form could be responsible for trapping two and possibly three atoms of ^{3}H. Three may not be out of the error range of the extrapolated value of Figure 1, 4.3, although this seems likely to be a minimum value: a 10% loss of labeled products through the operations of acid precipitation, ether extraction, DEAE column separation from [^{3}H]water, lyophilization, and HPLC separation would raise the value to 4.8. The most troubling aspect of the $-NH_3^+$ group hypothesis is the requirement that it behave like a monoprotonic group in the interconversion of citrate and isocitrate by virtue of restricted positional exchange in an active site that is presumed to accommodate extensive reorganization to permit anterofacial transfer of the proton, Scheme I.

A multiple pool of protons that fails to exchange through several cycles of catalysis is not easily explained in terms of water, per se. Exchangeable water is a common feature of the catalytic environment of proton-transferring enzymes. For example, the aldose–ketose isomerases always show some exchange of the transferred proton with medium (Rose, 1970). As an example of the exchange of active site water in the aconitase case, we have been unable to detect ^{18}O transfer in the reaction [2-^{18}O]-2-methylisocitrate + *cis*-aconitate → 2-methyl-*cis*-aconitate + isocitrate under conditions of intermolecular ^{3}H transfer.

A final explanation of the multiple proton pool is to suppose the existence of some element of structure, made up of proton-carrying amino acid residues, that either happens to be in the vicinity of the active site or preferably serves a role in the transfer of protons between water and the proton-donating base, a proton relay.

The concentration of *cis*-aconitate required for half-maximum trapping of E–^{3}H$^+$ derived from [^{3}H]water, 131 μM, can be used to estimate the average rate of dissociation of the enzyme-bound donor protons by the standard equation with corrections: the trapping of n atoms of ^{3}H will require n iterative actions of the substrate so that K_{tr} should be divided by n (3, 4, or 5) to calculate an intrinsic K_{tr} (=44, 33, or 26 μM). The conclusion that *identically* bonded protons are involved in each cycle is consistent with but may not be the only explanation for the linear character of the double-reciprocal plot, Figure 1. For example in a proton relay model, a common step, the transfer to water, may be rate limiting for exchange of any one of the hydrogens. In any case, it permits the use of K_{tr}/n for the calculation. The second correction is due to the isotope effect calculated by comparison of the partition of ^{3}H from [^{3}H]citrate between water and isocitrate (~4 to 1) compared with the partition of [^{1}H]citrate between *cis*-aconitate and isocitrate (1.8 to 1). It should require 1/2.2 as much *cis*-aconitate to trap a proton as a tritium on the enzyme.

Applying these corrections, it follows that ~131 μM/(3 to 5) × 2.2 or 20–12 μM *cis*-aconitate is required to trap half of one proton from this pool, which may include the protonated base itself depending on its pK relative to pH 7. From eq 1, with k_{cat}/K_m = 6 s^{-1}/15 μM, the proton exchange rate constant is in the range 5–8 s^{-1}. This can be compared with the steady-state maximum velocity at 0 °C of citrate → *cis*-aconitate, ~0.4 s^{-1}, determined from the rate of detritiation of [^{3}H]citrate × 2.2. The release of a single proton from the proposed multiproton pool is therefore ~12–20-fold greater than required to be considered rate limiting for the dehydration cycle and ~60-fold at 24 s^{-1} if the loss of any one of the four protons has the effect of completing the cycle $EH^+ \rightarrow E + H^+$.

The prospect of proton dissociation being slow, causing the accumulation of most of the enzyme in the form E–H$^+$, is made even less likely by the second unexpected phenomenon to be uncovered by this work: the stimulation by tricarboxylic acids other than *cis*-aconitate of the dissociation of the citrate-derived proton. The mechanism of this effect and its ability to influence the exchange of water-derived protons require further study. The interaction of citrate with E–H$^+$ could be substrate inhibitory if E·citrate derived by way of the citrate-dependent proton dissociation were nonfunctional. This is not the case since initial rate studies have not shown inhibition at high citrate. The ability of citrate to act with either E or E–H$^+$ may explain the invariance from pH 5 to pH 9 of V_{max} and V_m/K_m under initial rate conditions when both isocitrate and *cis*-aconitate are being generated (Schloss et al., 1984). The earlier considerations that noncompetitive inhibition patterns might be expected between a supposed *cis*-aconitate analogue, such as *trans*-aconitate and citrate, if the two reacted with different forms of the enzyme (E–H$^+$ and E, respectively) seem not to be justified. The correct form of inhibition is seen to be strictly competitive (Gawron & Jones, 1977; Ramsey et al., 1981).

Much more *cis*-aconitate was required to trap the proton derived from citrate than from the multiproton pool, suggesting a faster exchange rate for the former. Using eq 1 again, we calculate the exchange rate of the citrate-derived proton from K_{tr} of ~2.2 mM, Figure 2, corrected for a tritium isotope effect of 2.2. It should require 2.2 mM/2.2 = 1 mM to trap half of one citrate-derived proton before it exchanges with medium. This is 67 times greater than the K_m of *cis*-aconitate, indicating that proton dissociation is 67 times faster than the k_{cat} of 100 s^{-1}, i.e., 6700 s^{-1}. This is considerably more rapid than the 24-s^{-1} rate estimated for dissociation of a proton from the water-derived pool, a 275-fold difference. Although part of this difference can be attributed to the higher temperature of the [^{3}H]citrate experiments, there may be other explanations to consider. Clearly a proton relay model requires that the

relay not be less active than the action it is meant to explain. There are two qualifications one should consider in making this comparison. The pathways for trapping tritium with *cis*-aconitate in the two experiments differ. The ^3H derived from water does not pass through the central isomerization step of Scheme I in going to citrate + isocitrate. This could also be said for the citrate-derived ^3H if the bypass of that step is as simple as shown in that scheme. If, as seems likely, the bypass also includes an isomerization step, $E-H^+ \rightarrow E'-H^+$, then one or both of the isomerizations steps must be used when *cis*-aconitate traps a citrate-derived tritium into isocitrate. Only if one of the isomerization steps is fast could the value of $k_{cat} = 100\ s^{-1}$ be used to calculate k_{off} of the abstracted proton. Another factor that would increase the requirement for *cis*-aconitate in trapping ^3H from citrate is the presence of citrate itself at 0.1 mM, Figure 2. The sensitivity of the exchange to citrate concentration is not known. However, the dissociation constant of substrates for aconitase is reported to be $\sim 2\ \mu M$ (Schloss et al., 1984). This value most closely represents that of citrate since citrate is the major substrate in solution at equilibrium and E·citrate is the most abundant form of the enzyme at saturation (unpublished observation). Therefore at 0.1 mM citrate there could be a 50-fold exaggeration of K_{tr} of *cis*-aconitate in Figure 2.

The effects of temperature, a more circuitous path for trapping a citrate-derived proton into isocitrate, and citrate concentration on exchange need to be evaluated separately. The fact that they all serve to increase the dissociation of citrate-derived $E-H^+$ suggests that when corrections are made, the dissociation of the proton generated in the formation of *cis*-aconitate may be no greater than dissociation of a proton from the multiproton pool.

ACKNOWLEDGMENTS

Valuable contributions to this project in its early stages were made by Barry M. Levinson.

REFERENCES

Beinert, H. (1978) *Methods Enzymol. 54*, 435–445.

Beinert, H., Emptage, M. H., Dreyer, J.-L., Scott, R. A., Hahn, J. E., Hodgson, K. O., & Thomson, A. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 393–396.

Cleland, W. W. (1982) *Methods Enzymol. 87*, 390–405.

Crane, F. L., Glenn, J. L., & Green, D. E. (1956) *Biochim. Biophys. Acta 22*, 475–487.

Emptage, M. H., Kent, T. A., Kennedy, M. C., Beinert, H., & Münck, E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4674–4678.

Gawron, O., & Jones, L. (1977) *Biochim. Biophys. Acta 484*, 453–464.

Gawron, O., Waheed, A., Glid, A. J., & Jaklitsch, A. (1974) *Biochem. J. 139*, 709–714.

Glusker, J. P. (1971) *Enzymes (3rd Ed.) 5*, 413–439.

Kennedy, M. C., Emptage, M. H., Dreyer, J.-L., & Beinert, H. (1983) *J. Biol. Chem. 258*, 11098–11105.

Kent, T. A., Dreyer, J.-L., Kennedy, M. C., Huynh, B. H., Emptage, M. H., Beinert, H., & Münck, E. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1096–1100.

Lowry, O. H., Rosebrough, N. J., Farr, A. C., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Ramsey, R. R., Dreyer, J.-L., Schloss, J. V., Jackson, R. H., Coles, C. J., Beinert, H., Cleland, W. W., & Singer, T. P. (1981) *Biochemistry 20*, 7476–7482.

Rose, I. A. (1970) *Enzymes (3rd Ed.) 2*, 281–320.

Rose, I. A. (1977) in *Isotope Effect on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrup, D. B., Eds.) pp 209–232, University Park Press, Baltimore, MD.

Rose, I. A. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 50*, 361–395.

Rose, I. A. (1980) *Methods Enzymol. 64*, 47–59.

Rose, I. A., & O'Connell, E. L. (1967) *J. Biol. Chem. 242*, 1870–1879.

Rose, I. A., O'Connell, E. L., Litwin, S., & Bar Tana, J. (1974) *J. Biol. Chem. 249*, 5163–5168.

Ryden, L., Ofverstedt, L.-G., Beinert, H., Emptage, M. H., & Kennedy, M. C. (1984) *J. Biol. Chem. 259*, 3141–3144.

Schloss, J. V., Emptage, M. H., & Cleland, W. W. (1984) *Biochemistry 23*, 4572–4580.

Scholze, H. (1983) *Biochim. Biophys. Acta 746*, 133–137.

Schowen, K. B., & Schowen, R. L. (1982) *Methods Enzymol. 87*, 551–606.

Telser, J., Emptage, M. H., Merkle, H., Kennedy, M. C., Beinert, H., & Hoffman, B. M. (1986) *J. Biol. Chem. 261*, 4840–4846.

Thomson, J. F., Nance, S. L., Bush, K. J., & Szczepanik, P. A. (1966) *Arch. Biochem. Biophys. 117*, 65–74.

Villafranca, J. J. (1974) *J. Biol. Chem. 249*, 6149–6155.

Villafranca, J. J., & Mildvan, A. S. (1971) *J. Biol. Chem. 246*, 772–779.

Viola, R. E., & Cleland, W. W. (1978) *Biochemistry 17*, 4111–4117.