Carbon Kinetic Isotope Effects on the Spontaneous and Antibody-Catalyzed Decarboxylation of 5-Nitro-3-carboxybenzisoxazole

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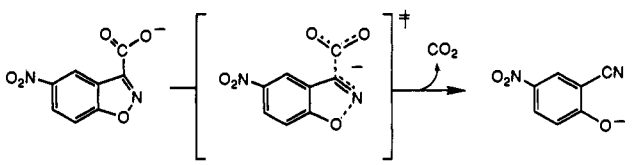
Abstract: The catalytic antibody 21D8 efficiently catalyzes the decarboxylation of a substituted 3-carboxybenzisoxazole—a simple, unimolecular reaction that is not susceptible to general acid/base catalysis but that is highly sensitive to the microenvironment. The transition-state structure of this decarboxylation reaction has been probed by measuring the carbon kinetic isotope effect for the uncatalyzed decarboxylation in water and for the dioxane-accelerated and antibody-catalyzed reactions. The isotope effect for the decarboxylation of 5-nitro-3-carboxybenzisoxazole is $k^{12}/k^{13} = 1.046$ in aqueous buffer at 20 °C and 1.048 for the antibody-catalyzed reaction. With increasing dioxane in the reaction medium, the rate of the spontaneous decarboxylation increases by almost four orders of magnitude, whereas the isotope effect displays only a slight, progressive decrease to $k^{12}/k^{13} = 1.043$ in 100% dioxane. Together, these results indicate that the structure of the transition state undergoes very little change in spite of $>10^4$-fold increases in the rate of the reaction caused by solvation of the substrate by organic solvents or the low dielectric environment of the antibody active site.

One mechanism of enzymic catalysis is the destabilization of charged substrates by extraction from aqueous solution into the low-dielectric environment of the protein binding pocket. Large rate accelerations may be achieved by desolvation of a molecule; for example, medium effects are believed to make an important contribution to the overall rate enhancements afforded by decarboxylases.[1] However, it has traditionally been difficult to measure the extent to which desolvation contributes to the catalytic efficiency of enzymes, because highly-evolved enzymes typically display complex catalytic mechanisms. For example, most decarboxylases use a cofactor such as pyridoxal phosphate, pyruvate, thiamine, or Mg^{2+} to effect catalysis.[6] Thus, most of the evidence for the import of desolvation has come from simple organic model systems. One reaction that is known to be particularly sensitive to solvation in organic solvents is the decarboxylation of substituted carboxybenzisoxazoles.[2]



This reaction has been thoroughly studied, and it is now well established that the transformation is a concerted, intermediateless process that proceeds through the charge-delocalized transition state shown in brackets. Most notably, the rate of the reaction is accelerated dramatically by transfer of the reactant from aqueous media to a dipolar aprotic solvent. For example, the rate of decarboxylation of 6-nitro-3-carboxybenzisoxazole in aqueous solution is accelerated by a factor of 10^4 by the addition of a benzonitrile phase,[2b] and the rate in neat hexamethylphosphoramide is 10^8-fold greater than that in water.[2a] In addition, intramolecular hydrogen bonding with the carboxylate anion (as occurs with 3-carboxy-4-hydroxybenzisoxazole) completely abolishes the reaction's sensitivity to solvent change, underscoring the important role of hydrogen bonding in controlling the decarboxylation rate. These and other data led Kemp and colleagues to attribute the solvent-promoted acceleration of the reaction to two opposing factors: first, the charge-delocalized transition state is stabilized in organic solvents by dispersion interactions, and second, the ground-state carboxylate is destabilized upon removal of hydrogen-bonding interactions with water.[2] In addition, others have noted the important influence of ion-pair formation in organic solvents.[3]

We recently reported the generation and characterization of a monoclonal antibody that catalyzes the decarboxylation of 5-nitro-3-carboxybenzisoxazole (shown above) with a 19 000-fold acceleration over the rate in aqueous buffer.[4] Several experiments indicated that the antibody behaves as a typical enzyme in displaying saturation kinetics and multiple turnovers. Because this decarboxylation reaction is known to be insensitive to general acid–base catalysis and stereochemical constraints, our results suggest that the large rate acceleration provided by the catalytic antibody can be ascribed almost entirely to medium effects. Fluorescence spectroscopy experiments using fluorescent hapten derivatives as reporter groups revealed that the protein binding site is very hydrophobic and is nearly completely inaccessible to water molecules when occupied. Thus, the binding pocket of this catalytic antibody provides a unique, rigid microenvironment that can be exploited to evaluate some of the specific factors that influence a protein's ability to desolvate and accelerate a chemical reaction, such as hydrogen-bond basicity, hydrogen-bond acidity, dipolarity, etc. As a first step, we have characterized the effect of solvation on transition-state structure by comparing the kinetic isotope effects of the spontaneous decarboxylation reaction in water and the dioxane-accelerated and protein-catalyzed reactions. Our ability to compare directly the characteristics of this simple model reaction in such disparate solvent environments presents a unique way to assess the significance of desolvation in protein-catalyzed decarboxylation reactions in a way that has heretofore been impossible.

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Experimental Section

Synthesis of 5-nitro-3-carboxybenzisoxazole from 2-nitrophenylacetic acid (Aldrich) was performed as previously described.[2a] Spectrophotometric grade 1,4-dioxane (Aldrich) was distilled from lithium aluminum hydride or sodium prior to use. The monoclonal antibody derived from the hybridoma cell line 21D8 was purified to homogeneity by affinity and ion-exchange chromatography.[4]

Kinetics. The rate of decarboxylation of 5-nitro-3-carboxybenzisoxazole in aqueous buffer (50 mM sodium phosphate, pH 6.5) or in buffer–dioxane mixtures was measured spectrophotometrically at 20 °C by monitoring formation of the product 2-cyano-4-nitrophenol at 380 nm. The rate of substrate decarboxylation in 100% dioxane at 20 °C was also measured spectrophotometrically using a stopped-flow spectrofluorimeter (HiTech Scientific); reactions were initiated with 3 mM tetramethylguanidine. The rate constants for the reactions were determined by fitting the data to the appropriate first-order rate equation.

Carbon Isotope Effects. The ^{13}C kinetic isotope effects for the decarboxylation were determined by monitoring the change in isotopic composition of the product CO_2 derived from 5-nitro-3-carboxybenzisoxazole, as previously described.[5] Reactions were carried out in 20 mL of 50 mM sodium phosphate (final pH 6.5) that had been freed of dissolved CO_2 by purging overnight with N_2 gas passed through an Ascarite trap. The uncatalyzed reactions were initiated by the addition of solid 5-nitro-3-carboxybenzisoxazole (190–250 μmol), which was quickly solubilized by brief sonication. If necessary, the pH of the mixture was adjusted to 6.5 by the addition of a small volume of 20% phosphoric acid through the sidearm of the reaction vessel, equipped with a stopcock and septum. The decarboxylations proceeded at 20 °C for the appropriate length of time before being quenched with concentrated phosphoric acid. The extent of the reaction, f (typically 10–20%), was determined in two ways: carbon dioxide was measured manometrically, and an aliquot of each reaction was withdrawn immediately before quenching for spectrophotometric quantitation of 2-cyano-4-nitrophenol. These independent determinations of extent of substrate conversion were in agreement.

The heavy-atom isotope effects for the antibody-catalyzed decarboxylation of 5-nitro-3-carboxybenzisoxazole were measured similarly, except that the reactions were initiated with antibody (50–70 nmol of immunoglobulin in 0.5 mL). Similarly, decarboxylations carried out in dioxane–buffer mixtures were initiated either with dioxane or with triethylamine. When dioxane was present as a cosolvent, the CO_2 was collected and purified by distillation in n-pentanol/liquid nitrogen traps (rather than the standard dry ice/2-propanol traps) to avoid contamination by the volatile cosolvent. In addition, each sample of purified CO_2 was scanned on the mass spectrometer to verify that no dioxane was present. Throughout these experiments, blank samples containing no substrate were routinely analyzed to verify that contamination by exogenous CO_2 was negligible.

The isotopic ratio of CO_2 produced from the low extent of conversion of 5-nitro-3-carboxybenzisoxazole was compared to that obtained from the complete conversion of reactant to products. For these experiments, 5-nitro-3-carboxybenzisoxazole was dissolved in dimethyl sulfoxide and reactions were initiated with excess triethylamine. Spectrophotometric and manometric measurement of total product formation verified that the decarboxylation had proceeded to completion; also, similar isotopic ratios were measured for all 100%-conversion reactions, including those that proceeded for 3 or 15 h. The isotopic ratio of CO_2 produced from all experiments was measured on a Finnigan Delta S isotope ratio mass spectrometer, and the carbon isotope effects were calculated according to eq 1, as previously described[5]

$$k^{12}/k^{13} = \log (1 - f)/ \log [1 - f(R_f/R_\infty)] \quad (1)$$

where the fraction of reactant conversion is represented by f, the isotopic ratio of CO_2 produced from a given fraction of reaction is R_f, and the isotopic ratio of the product after 100% conversion is equal to R_∞.

Results

The carbon isotope effects for the spontaneous, antibody-catalyzed, and solvent-accelerated decarboxylations of 5-nitro-3-carboxybenzisoxazole are shown in Table I. The isotope effect in 50% dioxane is derived from one experiment, and that in 15% dioxane is reported for two measurements; all other values report the average isotope effect (±1 standard deviation) obtained from 4–6 independent measurements. All reactions were carried out at 20 °C, and the amount of product produced during each reaction was in agreement with that predicted from the rate constants for decarboxylation (k_{obs} or k_{cat}) at 20 °C under the various conditions. The kinetic isotope effect on the spontaneous decarboxylation of 5-nitro-3-carboxybenzisoxazole at 30 °C, 1.0444 ± 0.0011, was slightly different from that measured at 20 °C.

Table I. Carbon Isotope Effects on the Decarboxylation of 5-Nitro-3-carboxybenzisoxazole at 20°C in 50 mM Sodium Phosphate Buffer (pH 6.5), Pure Dioxane, or Dioxane/Aqueous Buffer Mixtures

condition	k^{12}/k^{13}
uncatalyzed	1.0458 ± 0.0007
antibody-catalyzed	1.0484 ± 0.0009
15%[a] dioxane	1.0447[b]
30% dioxane	1.0453 ± 0.0012
50% dioxane	1.0441[c]
100% dioxane	1.0434 ± 0.0007

[a] Reported as % (v/v). [b] Average value of two determinations. [c] Single determination.

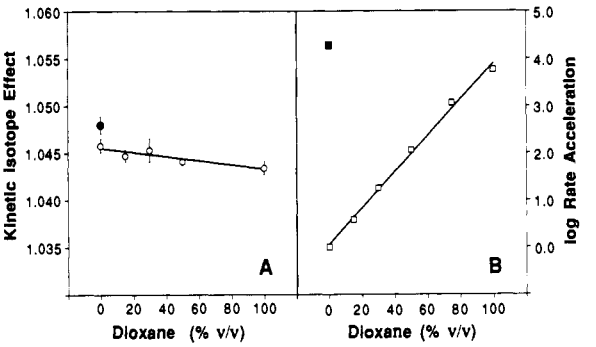

Figure 1. A: Effect of increasing dioxane concentration on the carbon kinetic isotope effect for the decarboxylation of 5-nitro-3-carboxybenzisoxazole in aqueous buffer (○). The kinetic isotope effect for the antibody-catalyzed decarboxylation is also shown (●). B: Effect of increasing dioxane concentration on the logarithm of the rate acceleration for the decarboxylation of 5-nitro-3-carboxybenzisoxazole ($k_{dioxane}/k_{H_2O}$, □). The logarithm of the rate acceleration for the antibody-catalyzed decarboxylation (■) was calculated as k_{cat}/k_{H_2O}, where k_{H_2O} is the first-order rate constant for the decarboxylation in aqueous buffer at 20 °C.

Increasing concentrations of dioxane resulted in dramatic increases in the rate of decarboxylation of 5-nitro-3-carboxybenzisoxazole, as depicted by a linear increase in the logarithm of the rate acceleration (right ordinate of Figure 1). Thus, the rate of decarboxylation of this carboxybenzisoxazole is increased by almost four orders of magnitude upon transfer from aqueous solution to 100% dioxane. This dioxane-dependent rate enhancement is consistent with Kemp's observations of the effect of aprotic organic solvents on the rates of decarboxylation of several substituted carboxybenzisoxazoles.[2] In spite of the substantial acceleration in the rate of decarboxylation, increasing the dioxane concentration caused only a slight progressive decrease in the carbon kinetic isotope effect (Table I and Figure 1). Furthermore, although 21D8 provides a rate acceleration of greater than four orders of magnitude (k_{cat} = 17 min^{-1}; $k_{H_2O} = 8.8 \times 10^{-4}$ min^{-1} in aqueous buffer at 20 °C), the carbon isotope effect on the protein-catalyzed reaction is, in fact, slightly higher than that on the uncatalyzed reaction (Table I and Figure 1).

Discussion

The magnitude of the carbon kinetic isotope effect on the spontaneous decarboxylation of 5-nitro-3-carboxybenzisoxazole of 1.046 lies within the expected range of carbon isotope effects (1.03–1.06) for decarboxylation reactions at room temperature,[7] suggesting extensive carbon–carbon bond cleavage in the transition state for the uncatalyzed reaction. The isotope effect for the decarboxylation of this carboxybenzisoxazole is substantially smaller than that reported for the decarboxylation of 4-pyridylacetic acid (1.057).[8] This result suggests that the transition state

(7) *Isotope Effects on Enzyme-Catalyzed Reactions*; Cleland, W. W.; O'Leary, M. H.; Northrop, D. B., Eds.; University Park Press: Baltimore, 1977; pp 233–250.

for the carboxybenzisoxazole decarboxylation occurs earlier along the reaction coordinate than does that for 4-pyridylacetic acid, a conclusion that is not surprising considering the enhanced reactivity of 5-nitro-3-carboxybenzisoxazole compared to 4-pyridylacetic acid.

The carbon isotope effect for the antibody-catalyzed decarboxylation of 5-nitro-3-carboxybenzisoxazole at 20 °C (k^{12}/k^{13} = 1.048) is slightly larger than the isotope effect we report for the uncatalyzed reaction. Thus, for this single-step reaction, the decarboxylation step appears to be entirely rate-limiting for the protein-catalyzed reaction, and the rates of substrate binding and dissociation are rapid compared to catalysis. The similarity of the isotope effects for the catalyzed and uncatalyzed reactions is noteworthy. Many enzymic decarboxylations display kinetic isotope effects substantially smaller than the intrinsic isotope effect (although the magnitude of intrinsic isotope effects is usually estimated rather than measured directly). Smaller isotope effects for enzyme-catalyzed reactions ordinarily reflect finite partitioning factors or commitments to catalysis.[9] However, exceptions do exist; for example, the oxidation of formic acid to carbon dioxide by formate dehydrogenase also involves only a single chemical step, and the enzyme-catalyzed reaction displays an isotope effect of k^{12}/k^{13} = 1.042.[10] Apparent intrinsic isotope effects have also been reported for orotidine-5′-monophosphate decarboxylase at low pH and temperature (k^{12}/k^{13} = 1.051 at pH 4.0, 0 °C)[11] and for the decarboxylation of the alternate substrate homoarginine by arginine decarboxylase (k^{12}/k^{13} = 1.061).[12] The similarity in magnitude of the isotope effects for the uncatalyzed and the protein-catalyzed decarboxylations of 5-nitro-3-carboxybenzisoxazole confirms the validity of using intrinsic isotope effects of model decarboxylation reactions as a reference point for evaluating partitioning factors for similar enzyme-catalyzed decarboxylations.

With increasing dioxane in the reaction medium, there results a dramatic increase in the rate of decarboxylation of 5-nitro-3-carboxybenzisoxazole, accompanied by only a slight decrease in the carbon kinetic isotope effect for the reaction. These results are similar to those observed for 4-pyridylacetic acid; the rate of decarboxylation of the latter acid increases almost 4000-fold in 75% dioxane compared to the aqueous reaction, in parallel with only a slight variation in the carbon kinetic isotope effect.[8] The absence of substantial variations in the carbon isotope effect for the decarboxylation of both 5-nitro-3-carboxybenzisoxazole and 4-pyridylacetic acid under these conditions suggests that solvation within a relatively apolar, aprotic environment, although leading to very large rate accelerations, does not cause large changes in transition-state structure. Such a conclusion reinforces previous suppositions that changes in solvation do not seriously affect the transition-state structure of enzyme-catalyzed decarboxylations. For example, this assumption has been invoked for the interpretation of partitioning factors for decarboxylation reactions catalyzed by 5′-orotidine monophosphate decarboxylase[11] and arginine decarboxylase.[12]

While the magnitude of the kinetic isotope effects makes it clear that there are no dramatic changes in the C–C bond order with changes in solvation, subtle changes in transition-state structure may explain the small variations in isotope effect observed under the different experimental conditions. Previous kinetic studies of carboxybenzisoxazoles are consistent with this interpretation. Kemp and Paul observed that the Hammett ρ values for decarboxylation of several substituted carboxybenzisoxazoles show little change over a broad range of solvents, despite a 10^6-fold variation in rate. However, the Hammett coefficient is anomalously low for water, the slowest solvent, and somewhat high for two of the fastest solvents (hexamethylphosphoramide and dimethylacetamide). Thus, solvation may alter transition-state structure to some extent, but these effects are likely to be small.

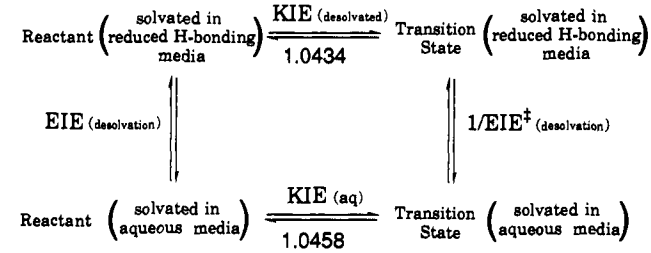

Figure 2. Equilibria between ground-state and transition-state species in different reaction media. KIE = kinetic isotope effect; $EIE_{desolvation}$ represents the equilibrium isotope effect for transfer of the ground state from an aqueous environment to a less-hydrogen-bonding medium; and $EIE^{\ddagger}_{desolvation}$ represents the equilibrium isotope effect for transfer of the transition state from water to a less-hydrogen-bonding medium.

The small variation in the isotope effects measured in water and dioxane may also reflect the differential solvation of the ground-state and/or the transition-state of the reaction in these different media. As the decarboxylation reaction proceeds, the reactant is transformed from a carboxylate anion, hydrogen-bonded and highly solvated in an aqueous environment, to a charge-delocalized transition state that is less capable of hydrogen bonding and more poorly solvated by water. The differences in solvation of these species upon changes in solvent may be reflected in an equilibrium isotope effect for transferring reactant or transition state from an aqueous medium to one of reduced hydrogen-bonding capacity. Assuming that the solvating power of the antibody binding pocket is similar to that of dioxane for this reaction (a reasonable assumption given the similar decarboxylation rates), the thermodynamic cycle in Figure 2 can be used to solve for these equilibrium isotope effects. The kinetic carbon isotope effect for the transformation of reactant to transition state within a medium of reduced polarity and hydrogen bonding is equal to k^{12}/k^{13} in 100% dioxane (1.0434). The transformation of reactant to transition state for the antibody-catalyzed reaction is described by the left- and upper-most reactions of Figure 2: the reactant is initially solvated in aqueous media and then transferred to the hydrophobic microenvironment of the protein binding site prior to conversion to the transition state. Therefore, the kinetic carbon isotope effect for the antibody-catalyzed reaction and the reaction in 100% dioxane can be used to determine that the equilibrium isotope effect for stripping the hydration shell from the ground state is 1.0048. Similarly, these values and the kinetic isotope effect for the uncatalyzed reaction in aqueous buffer can be used to calculate an equilibrium isotope effect for equivalent desolvation of the transition state ($EIE^{\ddagger}$) of 1.0025.

These values for the equilibrium isotope effects are quite reasonable. The charge-localized ground-state carboxylate anion is expected to be more stabilized in water than the charge-delocalized transition state; it is therefore not surprising that the equilibrium isotope effect for desolvation of the ground state is greater than the equilibrium isotope effect for desolvation of the transition state. Furthermore, these calculated isotope effects are of the appropriate magnitude for solvation of a carboxylate species. For example, the isotope effect reported for the solvation of CO_2 in water is 1.0011 at 30 °C.[13] A carbon isotope effect of 1.001–1.002 was attributed to the desolvation of the arginine carboxylate during binding by arginine decarboxylase.[12]

In summary, the carbon isotope effect on the decarboxylation of 5-nitro-3-carboxybenzisoxazole was measured for the uncatalyzed reaction in aqueous media, the dioxane-accelerated reaction, and the protein-catalyzed reaction. The isotope effects under all three conditions were similar, indicating that carbon–carbon bond

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cleavage is fully rate-limiting. Thus, the structure of the transition state for this decarboxylation does not change significantly under these three conditions. The results confirm the hypothesis that desolvation by a protein binding pocket or by organic solvents can cause a very large acceleration in the rate of a decarboxylation reaction but does not significantly alter the transition-state structure as reflected by the carbon isotope effect.

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Voltammetric Characterization of Rapid and Reversible Binding of an Exogenous Thiolate Ligand at a [4Fe-4S] Cluster in Ferredoxin III from *Desulfovibrio africanus*

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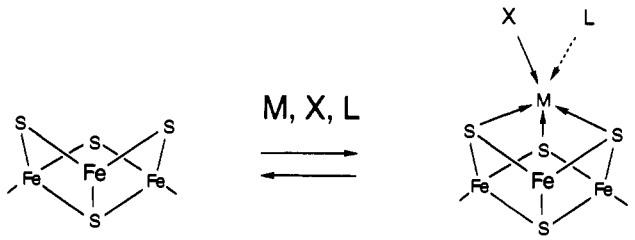
Abstract: Rapid and reversible, redox-coupled binding of an exogenous ligand to an Fe-S cluster in a protein has been studied by voltammetry. Ferredoxin III from *Desulfovibrio africanus* contains a [3Fe-4S] cluster whose transformation into a [4Fe-4S] center generates a new site for coordination. By coadsorbing with neomycin or polymyxin, an electroactive film of the protein is obtained at an edge-oriented pyrolytic graphite electrode which is then introduced to solutions containing the ligand. The equilibria and kinetics of ligation are determined by inspection and digital simulation of voltammograms measured over a range of scan rates. For solutions of 2-mercaptoethanol, results are consistent with coordination of the thiolate anion at both oxidized (dissociation constant = 28 μM) and reduced (dissociation constant = 97 mM) forms of the transformed [4Fe-4S] cluster. Direct detection of the thiolate-ligated reduced cluster by EPR is not feasible because the high concentration of mercaptoethanol that is required in solution results in cluster degradation. No interaction with thiolate is detected for the indigenous, stable [4Fe-4S] cluster or for the untransformed [3Fe-4S] precursor cluster. Under fast-scan conditions, the thiolate-ligated species appears as a trapped redox couple with $E^{\circ\prime}$ = -585 mV (cf. -396 mV for the native species) whereas, at a slow scan rate, equilibrium is established at all times and the observed reduction potential of the new couple depends upon thiolate concentration. At intermediate scan rates, a combination of trapped and dynamic, equilibrating species is observed. The great disparity in the equilibrium constants, reflected in the marked change in reduction potential, is attributed to a much faster rate of thiolate coordination to the *oxidized* cluster. An associative mechanism is proposed, involving nucleophilic attack by thiolate at the labile Fe subsite. The experiment provides a simple yet profound demonstration of time-separated coupling between electron transfer and ligand exchange (more generally extendable to conformational change) in a metalloprotein.

Introduction

Interest in Fe-S clusters has taken a new and unexpected direction with the realization that they have roles other than electron transfer.[1,2] They are now known to occur in several (de)hydratases,[3-6] the most thoroughly characterized of which is mitochondrial aconitase.[1,4-6] In its active form, aconitase contains a [4Fe-4S] cluster, one Fe of which is coordinated not to cysteine but to a hydroxide ion.[4,5] This 'subsite-differentiated' Fe is a Lewis acid catalyst, binding substrates (citrate, aconitate, isocitrate) and inhibitors,[5,6] but during aerobic isolation, it is released, resulting in formation of an inactive enzyme containing a [3Fe-4S] cluster.[1] The enzyme is reactivated by addition of Fe(II) under reducing conditions. A similar type of redox-linked [3Fe-4S] ↔ [4Fe-4S] transformation is known to occur for many other Fe-S proteins.[7-9] Moreover, metals other than Fe can readily be added to form heterometal [M3Fe-4S] clusters.[10-13] An important further development has occurred with characterization of the iron responsive element-binding protein (IRE-BP), a protein whose ability to bind to iron responsive elements (IRE's) located in mRNA is dependent upon the Fe status of the cell.[14] The discovery[15] that IRE-BP has close sequence homology with aconitase has suggested

Scheme I



that the Fe sensory mechanism is linked to some difference in the properties of [3Fe-4S] and [4Fe-4S] forms.[54] It has furthermore

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