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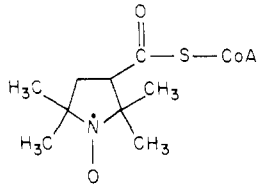
Interaction of a Paramagnetic Analogue of Oxaloacetate with Citrate Synthase†

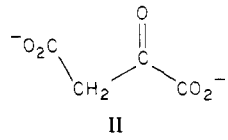
Stuart W. Weidman* and George R. Drysdale

ABSTRACT: Electron paramagnetic resonance studies have indicated that nitrosodisulfonate binds to pig heart citrate synthase. Titration of the enzyme with nitrosodisulfonate revealed several binding sites for the probe per subunit with one site ($K_D \approx 0.1$ mM) having a greater affinity than the others. The substrate, oxaloacetate, competed very effectively for one of the nitrosodisulfonate binding sites ($K_D < 10^{-2}$ mM) at the same time eliminating the weaker probe binding sites. Citrate and (*R*)- and (*S*)-malates also displaced the probe. Failure to resolve low- and high-field shoulders in the high gain–high modulation electron paramagnetic resonance spectra of the enzyme–nitrosodisulfonate system indicated that the bound probe was "weakly immobilized". However, the electron paramagnetic resonance spectrum of the bound probe changed to one typical of a "strongly immobilized" nitroxide upon the addition of a saturating concentration of the substrate acetyl coenzyme A (acetyl-CoA) to the enzyme–nitrosodisulfonate system, indicating the formation of a ternary acetyl-CoA–enzyme–probe complex. Titration of the acetyl-CoA saturated enzyme with the probe indicated one binding site per subunit ($K_D = 0.38$ mM). Thus, nitrosodisulfonate may be considered as a paramagnetic analogue of oxaloacetate in its interaction with citrate synthase. These results are compared with our previous studies with this enzyme, employing a spin-labeled acyl coenzyme A (acyl-CoA) derivative [Weidman, S. W., Drysdale, G. R., & Mildvan, A. S. (1973) *Biochemistry 12*, 1874–1883].

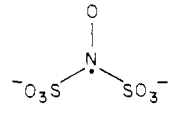
Spin-labeled coenzymes or substrates of enzymes have been utilized as active-site probes by several groups [for a recent review, see Morrisett (1976)]. We have previously examined the interaction of a spin-labeled analogue of acetyl-CoA (R·CoA,[1] I) with the enzyme citrate synthase (Weidman et al., 1973). These studies with R·CoA indicated that citrate synthase bound many di- and trinegatively charged organic ions which could be considered diamagnetic analogues of the substrates oxaloacetate (molecule II) or citrate. These observations aroused our interest in obtaining a paramagnetic analogue of oxaloacetate. Organic nitroxide analogues were not favored because of their large, steric bulk. Consideration of size, structure, and charge led to the choice of nitrosodisulfonate dianion (NDS, molecule III) as a possible analogue of oxaloacetate. Previous use of this substance in the field of biochemistry appears to have been limited to a reagent for modification of tyrosine and tryptophan residues in proteins (Cloughley et al., 1974). To our knowledge this paper is the first report of the application of NDS as an active-site enzyme probe.



R·CoA (I)



II



III

Experimental Procedure

Materials. Acyl-CoA esters were obtained from P–L Biochemicals. Using citrate synthase and oxaloacetate to convert acetyl-CoA or propionyl-CoA to citrate and methyl citrate, respectively, and analyzing the trimethyl esters by techniques previously described (Weidman & Drysdale, 1979),

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[1] Abbreviations used: R·CoA, 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy-CoA thioester; NDS, nitrosodisulfonate dianion.

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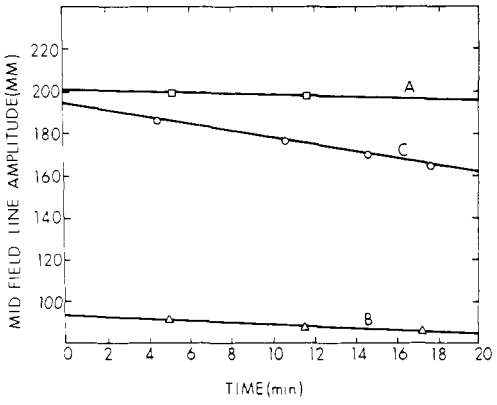

FIGURE 1: Decay of the midfield line of the paramagnetic resonance spectrum of NDS in solutions of (A) 40 mM Tris-HCl buffer, (B) buffer plus 0.11 mM citrate synthase sites, and (C) buffer plus 0.11 mM enzyme sites and 1.0 mM oxaloacetate.

we found <2% contamination of acetyl-CoA by propionyl-CoA and of propionyl-CoA by acetyl-CoA. Pig heart citrate synthase (Boehringer-Mannheim) was desalted and assayed as previously described (Weidman et al., 1973). Potassium nitrosodisulfonate (Fremy's salt) was obtained from Alfa Inorganics (Ventron Corp.) and stored in a desiccator over calcium oxide in the presence of ammonium carbonate in a separate dish to provide an ammoniacal atmosphere (Zimmer et al., 1971). The concentrations of NDS stock solutions were measured optically at 545 nm (ϵ = 20.8) (Murib & Ritter, 1952). All other materials were obtained from Sigma Chemical Co.

Electron Paramagnetic Resonance Studies. Spectra of 0.025–0.05-mL samples of NDS solutions were obtained in quartz capillaries by using a Varian E-3 spectrometer. The temperature was controlled at 25.5 ± 1.0 °C. Binding of NDS was measured by comparison of the peak-to-peak amplitude of the high-field line in the paramagnetic resonance spectrum of the sample containing the analogue plus enzyme with that of a comparable solution containing excess oxaloacetate to displace all bound NDS (both extrapolated back to the time of NDS addition to the sample). This comparison was necessary due to the slight broadening of the three lines of the unbound NDS caused by the increased viscosity of enzyme solutions compared to solutions of NDS in buffer alone.

Results

Stability of NDS in Enzyme Solutions. NDS is relatively stable in 40 mM Tris-HCl buffer at 24.5 °C (Figure 1, line A). However, the peak-to-peak line amplitude of NDS in solution containing citrate synthase decreases linearly with time over the period observed (Figure 1, lines B and C). The slow reduction in signal is presumably due to slow reduction of NDS by oxidizable amino acid residues in the enzyme. However, no loss in enzyme activity was observed when the enzyme was reassayed after the paramagnetic resonance experiments. This decrease in electron paramagnetic resonance signal with time necessitated the recording of three or more spectra over a period of 20 min, followed by plotting the peak-to-peak line amplitude as a function of time and extrapolation back to the time of NDS addition. The extrapolated line amplitude was used in binding calculations. That the slow reduction in signal in NDS–enzyme solutions is due to slow reduction of NDS by oxidizable amino acid residues on the enzyme and not due to time-dependent binding of the NDS to the macromolecule is indicated by the following observations: (1) the low- and high-field regions of the paramagnetic resonance spectrum of the NDS–enzyme system under conditions of high gain and modulation amplitude (Figure 2B) also decrease with time; the principal component of this region of the spectrum is the signal of bound NDS; and (2) when oxaloacetate is added to the NDS–enzyme system to displace bound NDS, the line amplitudes of unbound NDS return to the amplitudes expected when a correction is made for the percent signal lost during the period of observation.

NDS was actually somewhat protected from reaction with the enzyme when bound as compared to the unbound state. For example, under the conditions as given in the legend of Figure 2B, 14% destruction of signal was observed over the period of 20 min. On the other hand, when oxaloacetate was added to this system in a final concentration of 3 mM, 32% signal decay occurred over the same time period (all NDS is unbound under these conditions).

Binding of NDS to Citrate Synthase. A comparison of the electron paramagnetic spectrum of 0.356 mM NDS in buffer with that in buffer plus 0.241 mM citrate synthase [assuming

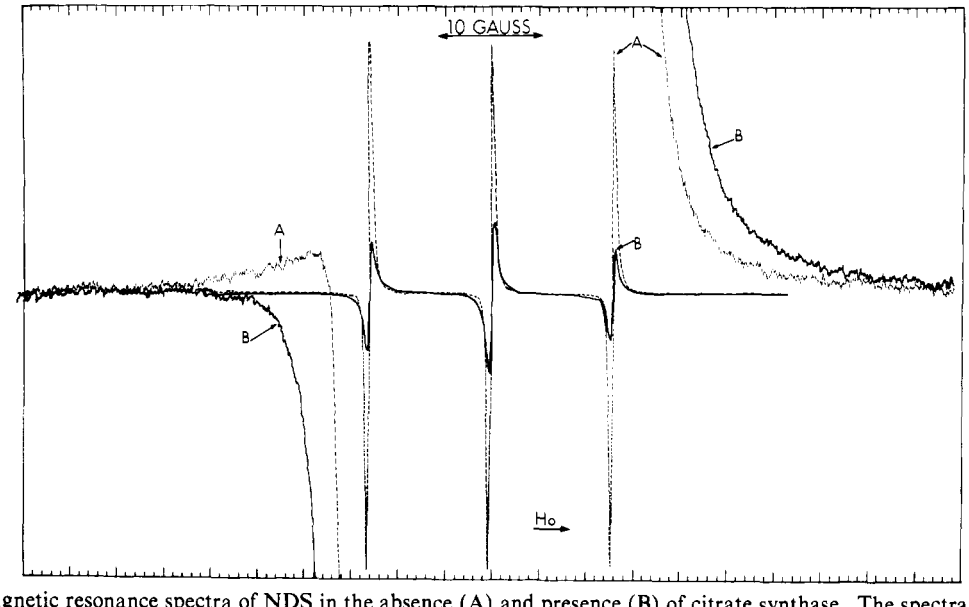

FIGURE 2: Paramagnetic resonance spectra of NDS in the absence (A) and presence (B) of citrate synthase. The spectra which are off-scale are higher gain (×125) and higher modulation amplitude (6.3 G) recordings of the on-scale (1.0-G modulation amplitude) spectra. In both spectra A and B the concentration of total NDS was 0.356 mM. The concentration of citrate synthase active sites in B was 0.482 mM.

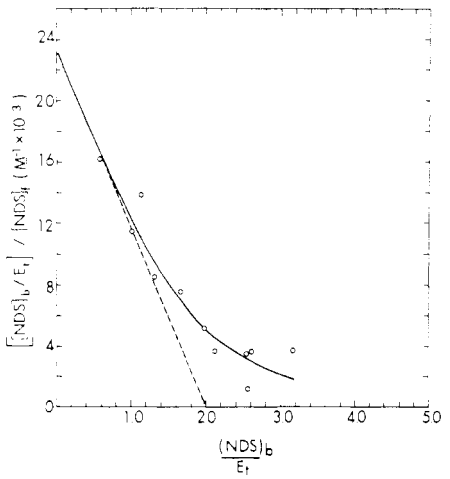

FIGURE 3: Scatchard plot of the paramagnetic resonance data for NDS binding to citrate synthase. The dashed line intersecting the abscissa at $(NDS)_b/E_t = 2.0$ was extrapolated from data obtained at low $(NBS)_b/E_t$ ratios.

96 000 daltons/molecule (Wu & Yang, 1970; Singh et al., 1970] indicated that the amplitudes of the three lines are decreased considerably by the addition of the enzyme (Figure 2). Such an observation is indicative of binding of the small molecule, NDS, to a large macromolecule. After applying a viscosity correction to the unbound NDS line amplitudes (see Experimental Procedure), we find that comparison of the amplitude of each line with that of the corresponding line in the standard yields unequal estimates for the percent unbound NDS. This observation indicates that the bound NDS spectrum contributes slightly to all three lines in the bound plus free spectrum and NDS is therefore only "partially immobilized" when bound. The "partially immobilized" nature of the bound NDS is consistent with the failure to observe shoulders or peaks in the low- and high-field regions of the spectrum under conditions of high gain and modulation amplitude. We expect the contribution of the bound radical to the observed spectrum to be smallest for the high-field line and have therefore used this line amplitude to estimate percent radical unbound.

The enzyme was titrated with NDS to determine the dissociation constant (K_D) of the radical–enzyme complex and the number of binding sites per enzyme molecule. A Scatchard plot of the data (Figure 3) was nonlinear, indicating at least two independent classes of specific binding sites or, more likely, two specific binding sites per enzyme dimer plus two, or more, nonspecific binding sites of lower affinity. By assuming two specific binding sites per dimer and extrapolating the data at low values of $(NDS)_b/E_t$ to an intercept on the ordinate, a value of 0.09 mM is obtained for the K_D of NDS from these two sites. Clearly the value of K_D obtained is an estimate only, since other straight lines intersecting the abscissa at slightly lower or higher values of $(NDS)_b/E_t$ than 2.0 could be drawn due to scatter of low values of $(NDS)_b/E_t$. From experiments involving displacement of bound NDS by oxaloacetate or citrate (see below), we believe that these two sites are the substrate binding sites. However, the argument that the ancillary sites may be substrate binding sites cannot be rigorously excluded at this time.

Displacement of NDS from Citrate Synthase by Substrates and Analogues. The addition of oxaloacetate in a concentration equivalent to the enzyme-active subunit concentration (48 000 daltons/subunit) to the NDS–enzyme system displaced slightly more than the equivalent concentration of bound NDS (Figure 4). This observation is consistent with the high

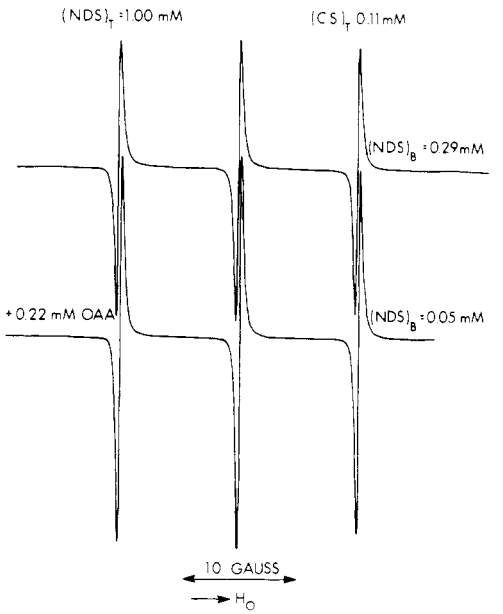

FIGURE 4: Paramagnetic resonance spectra of NDS in solutions of citrate synthase (CS) in the absence (top spectrum) and presence of oxaloacetate (OAA) (bottom spectrum). The concentration of citrate synthase is expressed as the dimer concentration. The gain and modulation amplitudes were the same for both spectra.

Table I: Apparent Dissociation Constants of Citrate Synthase Substrates and Analogues Determined by Competition with NDS

substrate or analogue[a]	K_D (mM)
oxaloacetate	*b*
citrate	0.2
(*R*)-malate	0.7
(*S*)-malate	3.0

[a] Added as Tris salt. [b] K_D too low to determine.

affinity of the enzyme for oxaloacetate determined by Srere (1966). The present studies indicate that there is one high-affinity binding site for oxaloacetate per enzyme subunit with a dissociation constant too low to measure accurately (<0.01 mM). That the added oxaloacetate displaced a slightly greater concentration of bound NDS than the oxaloacetate added is ascribed to a conformation change induced by bound oxaloacetate which eliminates the weaker NDS binding sites. The substrate, citrate, and the oxaloacetate analogues, (*R*)- and (*S*)-malates, also displaced enzyme-bound NDS. Titrations were performed with a less than saturating concentration of NDS and a variable concentration of analogue. The apparent dissociation constants determined from these competition studies are summarized in Table I.

Formation of the NDS–Citrate Synthase–Acetyl-CoA Ternary Complex. Our previous work with R·CoA showed that oxaloacetate formed a ternary oxaloacetate–enzyme–R·CoA complex when added to the R·CoA–enzyme system (Weidman et al., 1973). The affinity of the enzyme for R·CoA did not change detectably during the formation of the ternary complex, but the binding of oxaloacetate promoted a profound decrease in rotational freedom of the bound radical. When a saturating concentration of acetyl-CoA was added to the NDS–enzyme system, some of the bound NDS was displaced, but the motional freedom of the remaining bound NDS was severely restricted (Figure 5). The restriction of motion was indicated by the appearance of shoulders in the high- and low-field regions in the high gain–high modulation amplitude spectrum of the bound NDS in the presence of acetyl-CoA. The peak-to-peak distance between the low- and high-field

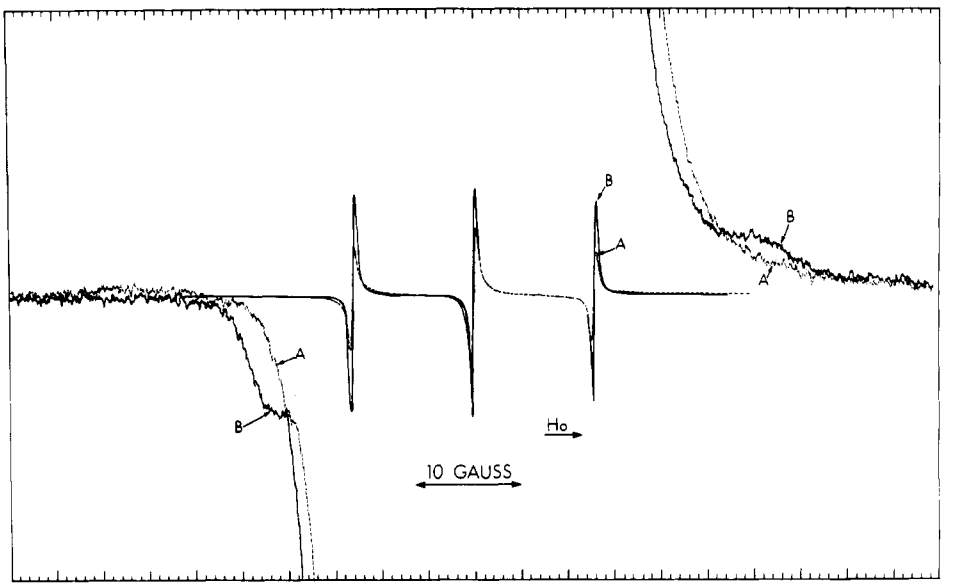

FIGURE 5: NDS–citrate synthase system in the absence (spectra A) and presence (spectra B) of acetyl-CoA (3.13 mM). The spectra which are off-scale are higher gain (×125) and higher modulation amplitude (6.3 G) recordings of the on-scale spectra. In both cases the concentrations of citrate synthase active sites and NDS were 0.482 and 0.356 mM, respectively.

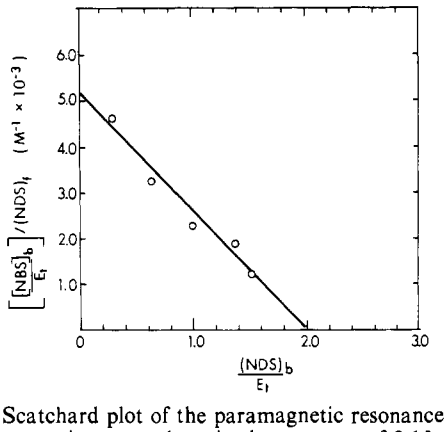

FIGURE 6: Scatchard plot of the paramagnetic resonance data for NDS binding to citrate synthase in the presence of 3.13 mM acetyl-CoA. The straight line fit to the data indicates two binding sites per citrate synthase dimer with K_D = 0.38 mM.

shoulders was 51 G (rigid glass limit = 57 G; Hamrick et al., 1972). We conclude from these observations that a ternary NDS–enzyme–acetyl-CoA complex was formed.

From the observation that a portion of the bound NDS was displaced by added acetyl-CoA, we postulated that there might be competition between NDS binding sites. Therefore, the enzyme–acetyl-CoA complex was titrated with NDS to determine whether the number of NDS binding sites was smaller relative to the number of corresponding binding sites for the free enzyme. The Scatchard plot of the binding data (Figure 6) was linear and now indicated just one NDS binding site per enzyme subunit with a dissociation constant for NDS of 0.38 mM. Thus, saturation of the enzyme with acetyl-CoA eliminated the weaker NDS binding sites present in the binary NDS–enzyme system.

The NDS–enzyme–acetyl-CoA complex was also observed to be protective with regard to probe signal destruction as compared to the NDS–enzyme–oxaloacetate system (100% unbound NDS). The decay of NDS in buffer plus acetyl-CoA solutions was accelerated slightly over that in buffer alone (7.5 vs. 2.5% in 20 min; NDS and acetyl-CoA concentrations were 0.36 and 3.13 mM, respectively).

When a saturating concentration of propionyl-CoA was added to the NDS–enzyme system, some of the probe was displaced, but the motional freedom of the remaining bound NDS was not changed over that in the absence of the propionyl-CoA. A ternary NDS–enzyme–propionyl-CoA complex was probably formed since oxaloacetate does form a ternary oxaloacetate–enzyme–propionyl-CoA complex (Weidman & Drysdale, 1973). However, the motional freedom of NDS in the NDS–enzyme–propionyl-CoA complex is apparently much higher than that in the corresponding acetyl-CoA ternary complex.

The possible formation of a ternary CoASH–enzyme–NDS complex could not be studied due to the reactivity of NDS with the sulfhydryl group of CoA. Since NDS is a strong oxidant, the reduction by CoASH was not unexpected. A similar reduction of organic nitroxides by free sulfhydryl groups under certain conditions has been noted by Morrisett & Drott (1969).

Discussion

Binding of NDS to citrate synthase appears to be specific since the radical is displaced by oxaloacetate and citrate, and, in addition, the substrate acetyl-CoA forms a ternary complex with NDS and the enzyme. We conclude therefore that NDS can be considered an analogue of oxaloacetate in its interactions with the enzyme.

We concluded that all of the bound NDS in the NDS–enzyme system was probably only "partially immobilized" because of the failure to observe shoulders on peaks in the low- and high-field regions of the paramagnetic resonance spectrum under conditions of high gain and modulation amplitude along with the observation of unequal estimates for the percent unbound NDS from the three lines in the unbound NDS spectrum (Figure 2B). However, the spectrum (Figure 2B) is of a mixture of bound and unbound NDS and therefore the spectrum of unbound NDS may be so dominant that it may not be possible to detect another "strongly immobilized" bound form of NDS if it exists. That the bound NDS appears to be "partially immobilized" may be indicative that the probe is bound through only one sulfonic acid group. However, "partial immobilization" might also result if NDS is loosely held to the enzyme through electrostatic interactions involving both sulfonate groups in a manner that allows rapid rotation about an axis joining both sulfur atoms.

The dissociation constant of oxaloacetate was too low to

measure accurately through competition with NDS by using the electron paramagnetic resonance spectrometer. Both Srere (1966) and Johansson et al. (1973) have reported tight binding of oxaloacetate to two sites per enzyme molecule (≤ 5 μM) in solutions containing urea. Steady-state kinetic studies by these two groups have provided similar estimates for the K_m for oxaloacetate (Johansson et al., 1973; Matsuoka & Srere, 1973). The binding of oxaloacetate to the enzyme in the presence of NDS eliminates the weaker binding sites for NDS on the enzyme. Srere (1966) has shown that a protein conformation change occurs when oxaloacetate binds. This enzyme conformational change could be responsible for the elimination of the weaker NDS binding sites.

The dissociation constant for citrate from the binary citrate–enzyme complex (0.2 mM) determined from competition with NDS is similar to the reported K_m for citrate at pH 6.1 in early kinetic studies on the pig heart enzyme (0.25 mM; Kosicki & Srere, 1961). Somewhat higher values have been reported (1–3 mM) at pH 7.4–8.2 by other investigators using different enzyme sources (Matsuoka & Srere, 1973; Johansson & Pettersson, 1974; Smith & Williamson, 1971). Weidman et al. (1973) reported that citrate formed a ternary R·CoA–enzyme–citrate complex and that the dissociation constant for citrate from this complex was 0.5 mM. The values determined for the dissocation constants of the oxaloacetate analogues, (*R*)- and (*S*)-malates, from their respective binary complexes with the enzyme are approximately a factor of 6 lower than their reported K_i values at pH 8.1 for the rat kidney enzyme (4.5 and 16 mM, respectively; Srere et al., 1973). However, the difference in magnitude between the two dissociation constants and their K_i values is comparable.

The addition of a saturating acetyl-CoA concentration to the enzyme–NDS system displaced a portion of the bound NDS, but the remaining bound radical was strongly immobilized. This profound change in bound NDS motional freedom was presumably caused either by a change in the mode of NDS binding so that both sulfonate groups participate, by a change in protein conformation caused by acetyl-CoA binding, or by steric interactions between the acetyl group of the CoA thioester and the nitroxide moiety of the NDS. This interaction could be the analogue of the acetyl-CoA carbanion attacking the carbonyl group of oxaloacetate. Therefore, the ternary NDS–enzyme–acetyl-CoA complex may be the analogue of a productive oxaloacetate–enzyme–acetyl-CoA ternary complex. The apparent elimination of weaker NDS binding sites on the enzyme by saturation with acetyl-CoA is consistent with our previous observation that some oxaloacetate and citrate analogues compete with R·CoA binding (Weidman et al., 1973) and with the inhibition studies of Johansson & Pettersson (1979). These sites could be the CoA–pyrophosphate binding regions on the enzyme.

Propionyl-CoA is a weak substrate for citrate synthase with a K_m similar to that for acetyl-CoA, but a $V_{max} \approx 10^{-4}$ that of acetyl-CoA (Weidman & Drysdale, 1973). The enzyme-catalyzed condensation of propionyl-CoA with oxaloacetate produces two diastereoisomers of methyl citrate, arising from attack on both *re* and *si* faces of the carbonyl of oxaloacetate (Brandange et al., 1977). A simple explanation for attack by propionyl-CoA on both faces of the oxaloacetate carbonyl would be that oxaloacetate can bind in productive ternary complexes with either its *re* or *si* face exposed to the incoming carbanion, by a 180° rotation for example. Thus, oxaloacetate does not appear to be held as tightly to the enzyme during methyl citrate production as it is during citrate formation (*si* attack only). The failure to observe a decrease in the motional freedom of NDS when a saturating concentration of propionyl-CoA was added to the NDS–enzyme system is consistent with the above arguments, if it is assumed that a ternary NDS–enzyme–propionyl-CoA complex was formed and it is an analogue of the analogous complex involving oxaloacetate.

Table II: Comparison of R·CoA and NDS Results[a]

R·CoA	NDS
Binary Complexes	
R·CoA partially immobilized	NDS partially immobilized
$K_D^{R\cdot CoA} = 0.1$ mM	$K_D^{NDS} = 0.09$ mM
2 binding sites/dimer	2.0 binding sites/dimer + other weaker affinity sites
Displacement by	
acetyl-CoA, CoASH } $K_D^{CoA} = 0.1$ mM	OAA, $K_D^{OAA} < 0.01$ mM
OAA and citrate analogues	citrate, $K_D^{citrate} = 0.2$ mM
Ternary Complexes	
OAA–CS–R·CoA	NDS–CS–acetyl-CoA
$K_D^{R\cdot CoA} = 0.1$ mM	$K_D^{NDS} = 0.4$ mM
R·CoA strongly immobilized	2 binding sites/dimer
	NDS strongly immobilized
citrate–CS–R·CoA	NDS–CS–CoASH
$K_D^{R\cdot CoA} = 0.2$ mM	study not possible
R·CoA strongly immobilized	

[a] CS = citrate synthase; OAA = oxaloacetate.

It is of interest to compare the results of enzyme studies using the R·CoA and NDS probes (Table II). The results obtained with the two probes complement each other nicely. Both form binary complexes with enzyme, and the bound probes are partially (weakly) immobilized. The addition of the other substrate leads to the formation of a ternary probe–enzyme–substrate complex in which the motion of the probe is greatly hindered. With R·CoA, formation of the ternary complex does not change the affinity of the enzyme for the probe, but with NDS there appears to be a fourfold decrease in enzyme affinity accompanying ternary complex formation. We (Weidman & Drysdale, 1973) and others (Johansson & Pettersson, 1977) have obtained evidence that the binding of one substrate (oxaloacetate) increases the affinity of the enzyme for the other substrate (acetyl-CoA) by a factor of ~20-fold. Neither of the paramagnetic probes can mimic this affinity change in their corresponding ternary probe–enzyme–substrate complexes, probably due to steric problems.

In conclusion, we feel that NDS is worthy of consideration as a general anion probe or as an active-site probe in compatible enzyme systems employing oxaloacetate, citrate, or structurally related anions as substrates or modifiers. The disadvantages of NDS are its reactivity with certain oxidizable amino acids and the sensitivity of the EPR line widths of unbound NDS to viscosity. The latter is an annoyance, but can be overcome by running an appropriate blank. However, the reactivity problem will be a function of the particular enzyme. The advantages of NDS as a probe are its small size compared to the organic nitroxides, the sensitivity of its paramagnetic resonance spectrum to molecular motion, and the extremely narrow lines which this molecule gives, due to the absence of ^{14}N–^{1}H coupling which produces the broader lines typicaly observed in most of the pyrrolidinyl or piperidinyl versions of spin-labels. We expect that it can be used to advantage in other enzyme systems.

Acknowledgments

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