Salt Inhibition of Nitrogenase Catalysis and Salt Effects on the Separate Protein Components†

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ABSTRACT: The nitrogenase activity of a 6:1 molar ratio of *Azotobacter vinelandii* Fe and MoFe proteins was investigated as a function of added salts at pH 7.5 and at 30 °C. Dinegative anions (Na_2HPO_4 and Na_2SO_3) are the most inhibitory. Mononegative anions are the next most inhibitory (NaI, NaBr, and NaCl). Except for LiCl, NaCl, and KCl, all other salts exhibited a rather simple inhibitory pattern. LiCl, NaCl, and KCl gave more complex inhibition patterns, indicating that specific cationic effects contribute to enzymatic inhibition. When properties of the separate proteins were studied, only the MoFe protein gave indications of salt interaction. A weak complex between Fe_{ox} and MoFe was observed which was stable in the presence or absence of 0.5 M NaCl. MgATP, $S_2O_4^{2-}$, and to a much smaller extent MgADP caused dissociation of this protein complex to occur.

Early studies using crude extracts or partially purified fractions of *Azotobacter vinelandii* nitrogenase reported that enzyme activity decreased with increasing salt concentration (Bulen & Lecomte, 1966). Later these same authors studied sedimentation behavior of the purified nitrogenase complex and reported that low ionic strength was essential for symmetrical sedimentation patterns.At concentrations greater than 0.1 M in NaCl, the nitrogenase complex formed multiple bands each sedimenting faster than the complex examined at lower NaCl concentrations (Bulen & Lecomte, 1972). Burns et al. (1970) measured the solubility of the purified MoFe protein as a function of NaCl concentration at 0 and 22 °C. In 0.015 M tris(hydroxymethyl)aminomethane (Tris), pH 7.4, and at NaCl concentrations near 0.05 M, the protein solubility approaches zero, and under these conditions, protein crystallization occurs. A series of 10 other salts was also examined, and results similar to those observed for NaCl were reported. Shah & Brill (1973) were unable to completely reproduce the results given by Burns et al. (1970) but were still able to crystallize the MoFe protein using the salt dilution method. More recently, Watt et al. (1980) have reported that the ability to oxidize the MoFe protein with organic dyes is influenced by the presence of NaCl in the protein.

The presence of NaCl or other salts in preparations of the nitrogenase complex or preparations of the component proteins clearly has an effect on the properties of *A. vinelandii* nitrogenase. The exact nature of this "salt effect" is not known, but its practical consequences are apparent in protein purification and crystallization schemes. This characteristic may be important to evaluate in more detail in order to gain a better understanding of nitrogenase function. For example, most activity-related studies where low protein concentrations are required are done at low NaCl connentrations in order to maximize enzymatic activity. However, most biophysical studies, requiring high protein concentrations, are carried out at high NaCl concentrations to maintain the protein solubility. The opportunity exists for conflicting results to be obtained or incorrect interpretations to develop from studies conducted at different salt concentrations where different protein properties may be expressed. In order to preclude such situations and to better understand the interaction of the nitrogenase proteins with their aqueous environment, we have studied the activity of the recombined protein components as a function of added salt and have also examined the behavior of the component proteins under these conditions.

Experimental Procedures

The nitrogenase proteins were prepared by the method of Burgess et al. (1980) and possessed activities of 2500 nmol of H_2/(mg·min) for the MoFe protein and 2300 nmol of H_2/(mg·min) for the Fe protein. Shethna protein (Shethna et al., 1968), also described as *A. vinelandii* iron–sulfur protein II (FeS II), was prepared from the nitrogenase complex (Bulen & LeComte, 1972) or from *A. vinelandii* crude extract by the method of Robson (1979). Standard H_2 evolution assays (Wherland et al., 1981) were used to assess the activity of the combined proteins as a function of increasing salt concentration. To 0.05 M *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), pH 7.5, were added various concentrations (0–0.5 M) of the salt to be examined following which the pH was readjusted to 7.5. The buffer containing the desired salt concentration was added to capped, anaerobic 10-mL assay vials, followed by addition of the components of the assay mixture. Assays in triplicate at each salt concentration were conducted for 10 min at 30 °C and quenched with 0.25 mL of trichloracetic acid, and the evolved H_2 was measured by gas chromatography. Enzyme activities at the various salt concentrations studied were referred to the normal assay containing no added salt which was defined as the 100% activity condition.

H_2 evolution assays at 0.1, 0.2, and 0.3, and 0.4 M NaCl were conducted as a function of time for at least 10 min to verify that enzyme activity was linear. Fixed time assays of 10 min were routinely used for determining the activity response of the nitrogenase components to the other salts studied. However, at least two selected salt concentrations were examined to verify a linear enzyme activity with time.

Fully active MoFe and Fe proteins in H_2O were prepared by passing $S_2O_4^{2-}$-reduced proteins through anaerobic G-25 columns equilibrated with H_2O. The MoFe protein sometimes streaks on the column, a result of partial crystallization as the NaCl concentration (originally 0.25 M) becomes diluted to

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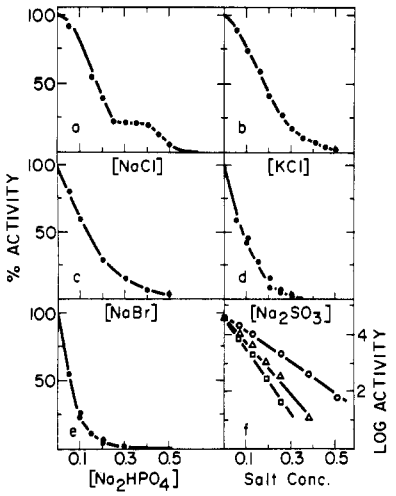

FIGURE 1: Nitrogenase activity as a function of added salt. Activity of a 6/1 molar (1.08 μM MoFe and 6.52 μM Fe) ratio of nitrogenase components at 30 °C and pH 7.5 as a function of increasing salt concentration for (a) NaCl, (b) KCl, (c) NaBr, (d) Na_2SO_3, and (e) Na_2HPO_4. (f) is the natural logarithm of the activity plotted against salt concentration for (□) Na_2HPO_4, (△) Na_2SO_3, and (○) NaBr.

~0.05 M due to dilution on the column. However, the protein is quite soluble in pure H_2O, and that portion of protein which crystallized on the column due to NaCl dilution eventually dissolves, causing the streak lines. The main band of protein passes easily through the column and is collected free of NaCl and $S_2O_4^{2-}$. The Fe protein passed smoothly through the H_2O-equilibrated columns. Optical and electron paramagnetic resonance (EPR) spectra, specific activity, and sedimentation measurements were carried out on these salt-free proteins as a function of buffer component [0.05 M TES, 0.05 M Tris, and 0.05 M *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes)] and salt (NaCl, NaBr, NaI, LiCl, KCl, $NaHSO_3$, and NaH_2PO_4) concentration. Microcoulometry (Watt, 1979) was used to assess the redox state of the proteins before and after spectroscopic and sedimentation measurements and to determine the presence or absence of $S_2O_4^{2-}$ in the protein solutions.

A Beckman Model E ultracentrifuge was used for sedimentation measurements. Protein solutions prepared as discussed above were studied as a function of concentration, and sedimentation values at zero protein concentrations were determined by extrapolation. Estimates of the concentration of protein components in mixtures were made by first running sedimentation patterns of the separate component proteins at known concentrations and then the mixture with components at the same concentration. The resulting sedimentation peaks were magnified 10-fold, traced on paper, and then integrated. By comparing the area of the separate components with that of the same component in the mixture, the relative proportion of each component protein in the mixture was determined.

Results

Protein Solubility. The MoFe protein from *A. vinelandii* is soluble to the extent of >100 mg/mL in 0.05 M Tris, pH 7.5, as long as the NaCl concentration equals or exceeds 0.25 M. At lower NaCl concentrations, protein solubility decreases rapidly (Burns et al., 1970) until at 0.05 M NaCl virtually complete protein insolubility is observed. However, we find that MoFe protein concentrations in excess of 10 mg/mL can easily be obtained in pure water by anaerobic Sephadex G-25 chromatography. Protein solutions prepared in this way are fully active and display spectroscopic properties nearly identical with those prepared in buffered NaCl solutions. Protein

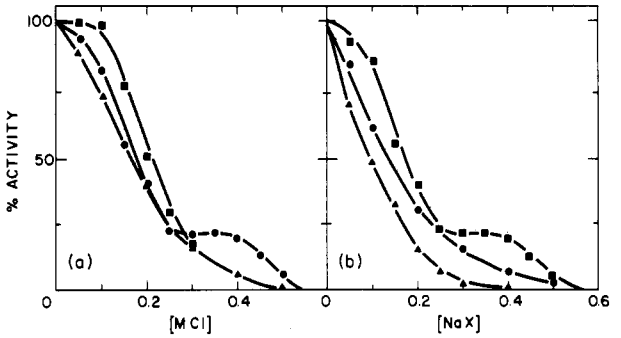

FIGURE 2: Salt component effects on nitrogenase activity. Same conditions as in Figure 1. (a) LiCl (■); NaCl (●); KCl (▲). (b) NaCl (■); NaBr (●); NaI (△).

crystallization occurs when Tris and NaCl are each made 0.05 M in MoFe solutions prepared in water, demonstrating that MoFe is soluble in the presence of high salt or in its complete absence but insoluble in dilute Tris–NaCl solutions.

Activity. Figure 1 is a plot of nitrogenase activity in the presence of the MgATP-generating system at an Fe/MoFe ratio of 6 as a function of added salt concentration for various salts. In this figure and Figure 2, 100% activity corresponds to specific activities of the MoFe protein ranging from 1300 to 1650 nmol of H_2/(min·mg), values typical for standard assay conditions at a 6/1 Fe/MoFe ratio. Corrections for the various salts required in the standard assay were not made, but rather these conditions were defined as the zero point on the abscissa scale in Figures 1 and 2. Values larger than zero correspond to the concentrations of salt added to the standard assay.

The H_2 evolution activity of nitrogenase is seen to be quite sensitive to salt concentration. Of the salts studied, phosphate and sulfite were the most inhibitory, completely inhibiting enzyme activity at 0.30 and 0.35 M, respectively. NaI, NaBr, KCl, and LiCl were the next most inhibitory in that order and gave incomplete inhibition at 0.40–0.60 M in salt. With the exception of LiCl, KCl, and NaCl, all other salts exhibited similar inhibition curves indicative of a single inhibitory process. This is shown in panel f of Figure 1 where a straight line is obtained throughout the entire range of salt concentrations studied when the natural logarithm of the remaining enzyme activity is plotted against the corresponding salt concentration.

Distinctly curved lines in contrast to the linear ones shown in panel f of Figure 1 were obtained for Na_2HPO_4 and Na_2SO_3 when the natural logarithm of the activity was plotted against the ionic strength created by these more highly charged ions. Ionic interactions other than those due to simple ionic strength effects seem to be involved in decreasing nitrogenase activity. This view is supported by the NaCl and KCl results in Figure 1 and by the more definitive results in Figure 2. This latter figure compares inhibitory effects of salts in which the cation is constant and the anion is varied with salts in which the cations are varied with a common anion. At a given salt concentration, the ionic strength is identical for all of these 1/1 salts studied, but clearly the activity responds differently to the various salt components. Figure 2a shows a general decrease of enzyme activity with increasing salt concentration for salts with chloride as the common anion but with variable cations (Li^+, Na^+, and K^+). LiCl is seen to be the least inhibitory with NaCl and KCl next in order up to a concentration near 0.27 M. At this concentration, all three salts inhibit to the same extent. Above 0.27 M, LiCl and KCl continue to inhibit identically. However, for NaCl from 0.25 to 0.40 M, a plateau of constant enzyme activity is evident.

Only above 0.40 M does increasing the NaCl concentration result in decreasing enzyme activity. We attribute the variation in activity seen in Figure 2a at a given salt concentration to specific cation interactions because all other variables were held constant.

Figure 2b shows the results of anion variation (Cl^-, Br^-, and I^-) in the presence of Na^+ as the common cation. A decrease in activity with increasing anion size is generally seen except for the distinctive plateau observed for NaCl. The effect of fluoride ion could not be determined because the Mg^{2+} of the assay mixture was precipitated as MgF_2.

The possibility was considered that the decrease in nitrogenase activity seen in Figures 1 and 2 was a result of salt inhibition of the creatine kinase generating system which would have the effect of decreasing MgATP availability and consequently lowering the nitrogenase activity. The linearity of nitrogenase activity with time for each point comprising the NaCl curve in Figure 1 and for selected salt concentrations for the other salts studied suggested that this was not occurring. This was substantiated by fixed-time assays (8 min) of nitrogenase components in the absence of the generating system but supplied with 5×10^{-3} M MgATP carried out as a function of NaCl concentration. Although assays conducted in this manner are not as sensitive and reproducible as those with the generating system, the results still showed the same general behavior shown in Figure 1 and demonstrated that the NaCl effect (and presumably the effect from other salts) was operating on the nitrogenase system. These latter results differed from those in Figure 1 only by showing a slight shift of the NaCl curve toward the right. The lower plateau began at 0.35 M NaCl and extended to 0.5 M before further inhibition occurred.

Spectroscopic Properties. The preparation of the MoFe and Fe proteins in water provides the opportunity to determine if buffer components or added salt perturbs the spectral properties of the proteins. The EPR signal of the MoFe protein in H_2O does not change shape or intensity when any of the buffer components are added or when the NaCl or NaBr concentration is increased from 0 to 0.5 M. The EPR signal of the Fe protein in H_2O is likewise unchanged.

Optical spectra of the MoFe protein in H_2O and in 0.05 M Tris, pH 8, containing 0.25 M NaCl were recorded as a function of protein concentration in the range 0.5–8 mg/mL (2.17×10^{-6} to 3.5×10^{-5} M). Plots of absorbance against concentration at 400 nm were linear in both cases, indicating that Beer's law is obeyed in this protein concentration range. Similar measurements carried out in 0.05 M Tris, pH 8, containing 0.25 M NaCl, where the protein is less soluble, showed positive deviations from the straight line at protein concentrations greater than 3.5 mg/mL. The deviation was corrected back to the projected straight line by the addition of NaCl, suggesting the deviation was due to the onset of crystallization at these higher protein concentrations. The slope of the straight line from the Beer's law plot in water was 7.5% smaller than that in 0.05 M Tris and 0.25 M NaCl, indicating that NaCl exerts a minor spectral perturbation.

The Fe protein was similarly studied from 0.5 to 10 mg/mL (7.8×10^{-6} to 1.56×10^{-4} M) in H_2O and 0.05 M Tris or TES and in 0.05 M Tris containing 0.25 M NaCl with no measurable differences being observed.

Ultracentrifugation. Sedimentation measurements of the Fe protein in H_2O and 0.05 M TES, pH 7.5, or in 0.05 M Tris containing 0.25 M NaCl, pH 8, gave $s_{20,w}$ values near 4.4 S which were essentially invariant of solvent type and only slightly concentration dependent in the range 1.0–10.0 mg/mL.

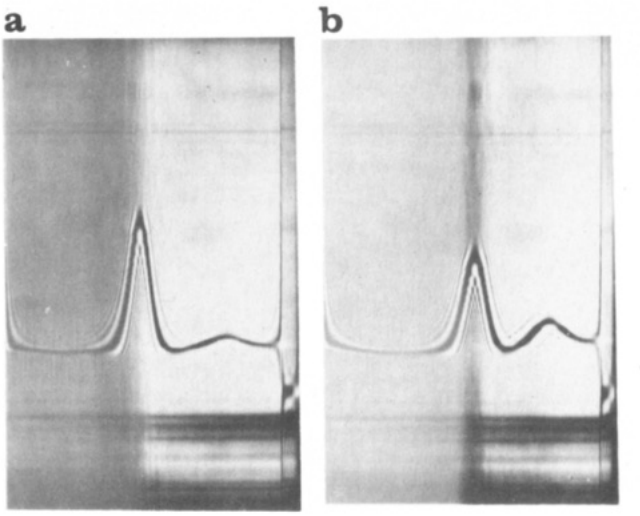

FIGURE 3: Ultracentrifugation of (a) reduced MoFe protein, 6.18 mg/mL (26.9 μM), and oxidized Fe protein, 1.81 mg/mL (2.87 μM), under argon in 0.05 M Tris and 0.25 M NaCl, pH 8.0, and (b) same conditions as (a) except 1×10^{-3} M MgATP is present. Both photographs were taken 32 min after the samples had attained a rotor velocity of 48 000 rpm.

Similar measurements for the MoFe protein in (1) 0.05 M Tris containing 0.25 M NaCl, pH 8, (2) 0.25 M NaCl, and (3) 0.05 M Tris gave $s_{20,w}$ values of 10.81, 10.75, and 10.42 S, respectively. These values are significantly higher than the $s_{20,w}$ values of 9.21–10.21 S for the MoFe protein in H_2O determined over the concentration range 2.0–9.0 mg/mL. The sedimentation values for the MoFe protein in the various solvents studied increase in the following order: $H_2O < 0.05$ M Tris < 0.25 M NaCl $\simeq 0.05$ M Tris and 0.25 M NaCl. The presence of NaCl seems to be a contributing factor in increasing the $s_{20,w}$ values of the MoFe protein.

The influence of NaCl concentration on complex formation in the three-component protein system consisting of the MoFe protein, the Fe protein, and the iron–sulfur II protein (FeS II) was also investigated. FeS II purified either from the nitrogenase complex or from crude extract had identical amino acid composition and Fe content, indicating two Fe atoms per 24 000 daltons. Aerobic or anaerobic preparations gave the same values. Reduction measurements by microcoulometry gave $E_{1/2}$ values of −250 mV vs. the normal hydrogen electrode (NHE) for a one-electron reduction per two Fe atoms, a result also consistent with $S_2O_4^{2-}$ uptake measurements carried out polarographically.

No complex formation was detected by ultracentrifugation measurements for a 1/1/1 mixture of the MoFe, Fe, and FeS II proteins in the range 3–10 mg/mL protein with or without 1×10^{-3} M $MgCl_2$ in (1) H_2O, (2) 0.05 M TES, pH 7.5, or (3) 0.05 M Tris and 0.25 M NaCl, pH 8. Exposure to O_2 of the 1/1/1 mixture with $MgCl_2$ present produced three discernible, rapidly sedimenting protein bands ($>10^6$ daltons) accounting for about 60% of the total protein with the remainder in the form of the free MoFe and Fe proteins.

Complex formation was detected under only one set of conditions. When reduced but $S_2O_4^{2-}$-free MoFe is mixed with one-electron-oxidized Fe protein (Fe_{ox}) in a 1/1 ratio, a molecular species is formed with an $s_{20,w}$ value near 11.5 S as shown in Figure 3a. Complete complexation does not occur under these conditions because there is also present a small protein peak with $s_{20,w} = 4.4$ S, indicative of Fe_{ox}, but at a concentration much lower than that expected for the total amount of Fe_{ox} protein present. From the area of the Fe_{ox} protein in this mixture and that for the same concentration

of Fe_{ox} in the free state, we estimate that 65% of the Fe protein is associated in the complex and 35% remains uncomplexed. By comparing the $s_{20,w}$ values of the larger peak and the Fe_{ox} peak and their relative areas as the concentration of the Fe_{ox} protein is varied at a fixed initial concentration of MoFe protein, we conclude that a weak 1/1 complex forms between the Fe_{ox} protein and the MoFe protein. This complex persists in H_2O or in 0.05 M Tris and 0.25 M NaCl, pH 8, but dissociates into its constituent proteins in either solvent when $S_2O_4^{2-}$ is added.

We have also investigated the effect of MgADP and MgATP on the formation of this 1/1 complex between reduced MoFe protein and Fe_{ox} protein. Under conditions where 65% of the Fe_{ox} is associated with MoFe, only 20% is associated in the presence of 1×10^{-3} M MgADP, and 0% is associated in the presence of 1×10^{-3} M MgATP. Dissociation in the presence of MgATP as shown in Figure 3b produces the free Fe_{ox} and MoFe proteins as evidenced by their sedimentation values.

The use of microcoulometry in characterizing the redox states of the proteins before and after these ultracentrifugation measurements was essential in confidently assigning the formation of the 1/1 complex to the interaction of Fe_{ox} and MoFe. Preliminary studies seemed to show that complex formation also occurred between the Fe protein and the MoFe protein in the presence of $S_2O_4^{2-}$. However, coulometric measurements clearly established that although $S_2O_4^{2-}$ was present when the ultracentrifuge cell was filled, not only was it absent following the sedimentation run but also the Fe protein had undergone a one-electron oxidation during this time interval. Activity measurements after ultracentrifugation and other controls excluded the possibility that O_2 had consumed the $S_2O_4^{2-}$ and oxidized the Fe protein. The explanation for these observations is that the Fe protein decomposed the $S_2O_4^{2-}$ present and underwent spontaneous "self-oxidation" (Stephens et al., 1981) during the run, resulting in complex formation between the resulting Fe_{ox} and MoFe proteins. When these binding measurements were repeated and $S_2O_4^{2-}$ was verified by microcoulometry to be present both before and after ultracentrifugation, then two peaks were observed, and no evidence for complexation was found.

Discussion

A. vinelandii nitrogenase activity is strongly inhibited by the presence of ionic electrolytes. Large negative anions exert the largest inhibitory effect as evidenced by comparing in Figures 1 and 2 the decreasing inhibitory ability, at a given salt concentration (with a common cation present), of sodium phosphate (70% in the HPO_4^{2-} form), sodium sulfite (78% in the SO_3^{2-} form), NaI, NaBr, and NaCl at pH 7.5. The larger more negative anions clearly display the greater inhibition. Cation effects were not extensively investigated because (1) divalent Mg^{2+} cations are required for MgATP hydrolysis and the presence of a second cation could interfere with this process by competing with Mg^{2+} for ATP and (2) multivalent cations cause protein precipitation. For example, $MgCl_2$ at 0.02 M is used for protein precipitation during protein purification (Bulen & LeComte, 1972), and complete precipitation of MoFe or Fe proteins occurs at $< 5 \times 10^{-4}$ M $LaCl_3$ (G. D. Watt, unpublished results). However, cation effects are clearly operative as seen by comparing the inhibition curves in Figure 2 for LiCl, NaCl, and KCl where the anion is held constant. The larger K^+ cation causes greater inhibition. We attribute the plateau in the NaCl curve to be a consequence of Na^+ because this plateau is absent for corresponding conditions when K^+ and Li^+ are the only cation present. The sodium effect which occurs with the nitrogenase system may be related to the sodium activation requirement reported by Apte & Thomas (1980) for cyanobacteria nitrogenase.

The interaction of electrolytes with macromolecular polyelectrolytes such as proteins is quite complex. The site of interaction of salts with nitrogenase is not known nor can much detail be inferred from the results presented here. The minor spectral perturbation of the MoFe protein with NaCl, the crystallization of this protein by NaCl dilution, and the small effect of NaCl on the sedimentation value suggest this protein may be responsible for at least some of the NaCl inhibition. Furthermore, we have noted (Watt et al., 1983) that the rate of oxidation of the MoFe protein by organic oxidants is influenced by the presence of NaCl, again suggesting a NaCl effect specific to this protein.

Instead of a single specific salt interaction with one of the component proteins, however, it is possible that increasing the salt concentration prevents the constituent proteins from interacting during the catalytic cycle, and by this process, added salt decreases the nitrogenase activity. This is difficult to test directly, and the experiments reported here have simply attempted to show whether or not component protein interaction occurs in the presence or absence of added salt in the absence of enzyme turnover. A previous report on this subject from our laboratory has appeared (Wang et al., 1985) and indicates that no nitrogenase complex forms under the conditions studied. We have extended these previous studies to include a wider NaCl and component protein concentration range, and we have also examined in further detail the effect of FeS II, prepared either from *A. vinelandii* crude extract or directly from the nitrogen complex. The results reported here confirm those of our previous studies (Wang et al., 1985) in showing that no complex is formed from a 1/1/1 mixture of reduced MoFe, Fe, and FeS II proteins in the presence or absence of added NaCl.

A weak 1/1 complex is formed, however, between MoFe and Fe_{ox} which indicated no tendency to dissociate even at elevated NaCl concentrations. Dissociation occurs only when $S_2O_4^{2-}$, MgADP, or MgATP is added. Eady (1973) and Thorneley et al. (1975) have previously observed the formation of a 1/1 complex between the *Klebsiella pneumoniae* and *Azotobacter chrooccum* proteins in the absence of $S_2O_4^{2-}$, which dissociated in the presence of $S_2O_4^{2-}$ into the two component proteins. The degree of oxidation of the Fe proteins in these complexes was not determined by Eady (1973) and Thorneley et al. (1975), and it is likely that inadvertent oxidation or self-oxidation could have occurred and that the complexes observed are the same as that reported here. If this is so, the *K. pneumoniae* components seem to form a slightly stronger complex. Our results clearly show that NaCl does not dissociate the complex once it is formed and it appears that the presence or absence of NaCl has no effect on the interaction of the component proteins, at least in the absence of enzyme turnover.

The complete dissociation of MoFe–Fe_{ox} by MgATP but only a partial dissociation by MgADP indicates that the conformation of Fe_{ox} differs in the presence of the two nucleotides. The reaction of MoFe–Fe_{ox} with $S_2O_4^{2-}$ also completely dissociates the complex and suggests that a significant difference exists between Fe_{ox} and Fe_{red}. These observations are consistent with the known properties (Mortenson & Thorneley, 1979) of the isolated Fe protein in its two oxidation states and in the presence of the two nucleotides.

There is some resemblance between the MoFe–Fe_{ox}-$(MgADP)_2$ complex which we report here and that postulated

by Thorneley & Lowe (1983), $MoFe_{red}$–$Fe_{ox}(MgADP)_2$, purportedly resulting from the transfer of an electron from $Fe_{red}(MgATP)_2$ to MoFe. Thorneley & Lowe (1983) suggest that the dissociation of $Fe_{ox}(MgADP)_2$ from the latter complex is rate limiting during nitrogenase turnover with an off rate constant of 6.4 s^{-1}. The weak binding of $Fe_{ox}(MgADP)_2$ to MoFe reported here bears some similarity to that of the Thorneley and Lowe complex even though in our complex the MoFe protein is one electron more oxidized compared to that in their complex.

Registry No. LiCl, 7447-41-8; NaCl, 7647-14-5; KCl, 7447-40-7; NaI, 7681-82-5; NaBr, 7647-15-6; Na_2HPO_4, 7558-79-4; Na_2SO_3, 7757-83-7; nitrogenase, 9013-04-1.

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Symmetry and Asymmetry in Mandelate Racemase Catalysis†

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ABSTRACT: Kinetic properties of mandelate racemase catalysis (V_{max}, K_m, deuterium isotope effects, and pH profiles) were all measured in both directions by the circular dichroic assay of Sharp et al. [Sharp, T. R., Hegeman, G. D., & Kenyon, G. L. (1979) *Anal. Biochem. 94*, 329]. These results, along with those of studying interactions of mandelate racemase with resolved, enantiomeric competitive inhibitors [(*R*)- and (*S*)-α-phenylglycerates], indicate a high degree of symmetry in both binding and catalysis. Racemization of either enantiomer of mandelate in D_2O did not show an overshoot region of molecular ellipticity in circular dichroic measurements upon approach to equilibrium. Both the absence of such an overshoot region and the high degree of kinetic symmetry are consistent with a one-base acceptor mechanism for mandelate racemase. On the other hand, results of irreversible inhibition with partially resolved, enantiomeric affinity labels [(*R*)- and (*S*)-α-phenylglycidates] reveal a "functional asymmetry" at the active site. Mechanistic proposals, consistent with these results, are presented.

Stereospecificity of enzymic catalysis is a key concept in enzymology; that is, except in the case of the interconversion of stereoisomers, enzymes generally process only one enantiomer. Such stereospecificity is not at all surprising since enzymes are proteins composed only (*S*)-amino acids and therefore are inherently asymmetric. Racemases, because of their ability to bind and catalyze either of two enantiomers, present a paradox: How are the groups at the active site so arranged as to catalyze a symmetrical reaction within an inherently asymmetrical environment?

Thus far, there are only limited experimental data for racemases to help answer this question. Although some researchers have reported the K_m and V_{max} values in both directions for racemases, the question of enantiomeric catalysis and binding has been explored in detail only for alanine racemase. Thus, Wang & Walsh (1978, 1981) systematically probed the active site of alanine racemase by using the *R* and *S* enantiomers of both β-chloro- and β-fluoroalanines as so-

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