The Binding of Reduced Nicotinamide Adenine Dinucleotide to Citrate Synthase of *Escherichia coli* K12†

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ABSTRACT: Citrate synthase from *Escherichia coli* enhances the fluorescence of its allosteric inhibitor, NADH, and shifts the peak of emission of the coenzyme from 457 to 428 nm. These effects have been used to measure the binding of NADH to this enzyme under various conditions. The dissociation constant for the NADH–citrate synthase complex is about 0.28 μM at pH 6.2, but increases toward alkaline pH as if binding depends on protonation of a group with a pK_a of about 7.05. Over the pH range 6.2–8.7, the number of binding sites decreases from about 0.65 to about 0.25 per citrate synthase subunit. The midpoint of this transition is at about pH 7.7, and it may be one reflection of the partial depolymerization of the enzyme which is known to occur in this pH range. A gel filtration method has been used to verify that the fluorescence enhancement technique accurately reveals all of the NADH molecules bound to the enzyme in the concentration range of interest. NAD^+ and $NADP^+$ were weak competitive inhibitors of NADH binding at pH 7.8 (K_i values greater than 1 mM), but stronger inhibition was shown by 5′-AMP and 3′-AMP, with K_i values of 83 ± 5 and 65 ± 4 μM, respectively. Acetyl-CoA, one of the substrates, and KCl, an activator, also inhibit the binding in a weakly cooperative manner. All of these effects are consistent with kinetic observations on this system. We interpret our results in terms of two types of binding site for nucleotides on citrate synthase: an active site which binds acetyl-CoA, the substrate, or its analogue 3′-AMP; and an allosteric site which binds NADH or its analogue 5′-AMP and has a lesser affinity for other nicotinamide adenine dinucleotides. When the active site is occupied, we propose that NADH cannot bind to the allosteric site, but 5′-AMP can; conversely, when NADH is in the allosteric site, the active site cannot be occupied. In addition to these two classes of sites, there must be points for interaction with KCl and other salts. Oxaloacetate, the second substrate, and α-ketoglutarate, an inhibitor whose mode of action is believed to be allosteric, have no effect on NADH binding to citrate synthase at pH 7.8. When NADH is bound to citrate synthase, it quenches the intrinsic tryptophan fluorescence of the enzyme. The amount of quenching is proportional to the amount of NADH bound, at least up to a binding ratio of 0.50 NADH per enzyme subunit. This amount of binding leads to the quenching of 53 ± 5% of the enzyme fluorescence, which means that one NADH molecule can quench all the intrinsic fluorescence of the subunit to which it binds.

Weitzman (1966a) reported that citrate synthase from acetate-grown *Escherichia coli* K12 is strongly inhibited by NADH. Practically 100% of the activity of this enzyme could be inhibited by 0.2 mM NADH in a standard assay, at pH values below 7.5 (Weitzman, 1966b). Other *E. coli* enzymes which are allosterically inhibited by NADH have since been discovered (Sanwal, 1969; Sanwal and Smando, 1969; Wright and Sanwal, 1969; Suzuki et al., 1969). Sanwal (1970) has proposed that NADH is a major regulator for energy-producing pathways in enteric bacteria.

Wright and Sanwal (1971) studied the binding of NADH to *E. coli* citrate synthase by equilibrium dialysis. They found the binding to be very weak, though somewhat improved by the presence of one of the substrates, acetyl-CoA. Even with acetyl-CoA present, the binding was too weak to account for the inhibition observed in kinetic experiments. Moreover, it was puzzling to find that acetyl-CoA improved the binding, since NADH inhibition can be largely overcome in assays by increasing the concentration of acetyl-CoA (Weitzman, 1966a; Wright and Sanwal, 1971).

We have discovered that *E. coli* citrate synthase enhances the fluorescence of NADH. This fact permits the use of fluorescence techniques, which are rapid and require little enzyme, to examine in detail the binding interaction between the enzyme and the reduced coenzyme. The results of our NADH fluorescence binding measurements on *E. coli* citrate are reported in this paper.

Experimental Section

Enzyme. Citrate synthase from acetate-grown *E. coli* K12 was prepared exactly as described by Tong and Duckworth (1975). Three different preparations were used in this work. That of January 1974 had a specific activity of 26 U/mg in the assay described previously (Tong and Duckworth, 1975); that of April 1974 had a specific activity of 18; and that of October 1974 had a specific activity of 44. The three preparations all appeared to be homogeneous by a number of criteria, and all gave essentially the same binding results for NADH. We have noted previously that specific activity by itself is not a trustworthy criterion for judging the purity of this enzyme, and that different preparations of apparently equal, high purity can have very different specific activities (Tong and Duckworth, 1975). A given batch of enzyme retained the same NADH binding properties for at least 6 months after preparation, if stored near 4°C in 0.02 M Tris-Cl–0.05 M KCl, at pH 7.8.

Reagents. Oxaloacetic acid, and NADH, disodium, were obtained from Boehringer. Solutions of NADH were made

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up fresh daily, and the concentrations were determined by the A_{340}, using ϵ_M 6220 M^{-1} cm^{-1}. Acetyl-CoA was from PL Biochemicals. α-Ketoglutaric acid, 3′- and 5′-AMP, NAD^+, $NADP^+$, and NADPH were all from Sigma. Other chemicals were reagent grade.

Fluorescence Enhancement Measurements and Calculations. All fluorescence measurements were performed at room temperature (21 ± 1°C) on an Aminco-Bowman spectrophotofluorometer, using 1-cm cuvettes. Relative intensities of fluorescence were read directly from the photometer, and corrected for the blank fluorescence in the absence of NADH. The fluorescence enhancement, λ, where

$$\lambda = F_{(\text{complex})}/F_{(\text{free})}$$

was determined by measuring the fluorescence of a small amount of NADH, $F_{(\text{free})}$, and then titrating this with large amounts of enzyme measuring the fluorescence, $F_{(\text{enz})}$, after each addition. A plot of $(F_{(\text{enz})} - F_{(\text{free})})^{-1}$ vs. [enzyme]$^{-1}$ is linear as long as [enzyme] ≫ [NADH], and it yields $(F_{(\text{complex})} - F_{(\text{free})})^{-1}$ as intercept on the vertical axis, from which λ, the enhancement, may be calculated. The values of λ used in the binding calculations were determined for emission at 428 nm, since NADH bound to citrate synthase has its maximal fluorescence emission at that wavelength, unlike free NADH for which the maximum is at 457 nm. Excitation in all experiments was at 340 nm.

Fluorescence data were corrected for the absorption of excitation light by added ligands (especially NADH itself), and concentrations of free and bound NADH were calculated by using the relationships:

$$F_o^{\text{corr}} = F_{N,f}([\text{NADH}]_{\text{bound}}\lambda + [\text{NADH}]_{\text{free}})$$

and

$$[\text{NADH}]_{\text{total}} = [\text{NADH}]_{\text{free}} + [\text{NADH}]_{\text{bound}}$$

where F_o^{corr} is the corrected fluorescence of a solution of NADH and enzyme at 428 nm, and $F_{N,f}$ is the fluorescence of a 1 μM solution of free NADH at 428 nm under our conditions.

The fluorescence of free NADH is linear with concentration, even after the above correction has been made, only at rather low concentrations (<10 μM). This nonlinearity has been noted by Dickinson (1970) among others. We corrected for the nonlinearity, when it was necessary, by using an empirical relationship between $F_{N,f}$ and [NADH] in the absence of citrate synthase.

Gel Filtration Binding Studies. The principle of Hummel and Dreyer (1962) was used. Columns of Sephadex G-50-80, 1.0 × 80 cm approximately, were equilibrated with solutions of about 20 μM NADH in 0.02 M Tris-Cl (pH 7.86) or 0.015 M sodium phosphate (pH 7.00) by passing at least 80 ml of the equilibration buffer through each column. The exact concentrations of NADH for particular experiments were determined from the A_{340} of the solutions, using ϵ_M 6220. A sample of citrate synthase, a known amount close to 40 nmol of subunits dissolved in 1.00 ml of equilibration buffer, was loaded on a column, and 0.99-ml fractions were collected, at room temperature. The NADH content of each fraction could be measured most precisely by determining F_{460}, the fluorescence at 460 nm (excitation at 340 nm), using a calibration curve for F_{460} vs. [NADH] obtained in 0.99-ml portions of the same Tris-Cl or phosphate buffer. The area of the trough in the elution profile (see Figure 4) has units of nanomoles of NADH when [NADH] is expressed in nanomoles milliliter^{-1} and fraction volume in milliliters, and it is equal to the amount of NADH bound to the enzyme (see Hummel and Dreyer, 1962). The area of the peak, however, is equal to this same amount of NADH multiplied by λ_{460}, the enhancement of NADH fluorescence (at 460 nm) when NADH is bound to the enzyme, since this peak is due entirely to enzyme-bound NADH. As will be seen, 20 μM NADH is enough almost to saturate the enzyme binding sites, assuming that the binding constants, determined at pH 7.00 and 7.86 by the fluorescence enhancement method, are correct.

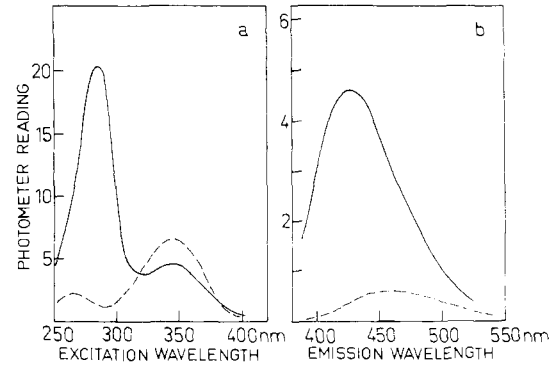

FIGURE 1: The effect of *E. coli* citrate synthase on the fluorescence excitation and emission of NADH. (a) Excitation spectra. The broken line is for free NADH, 1.94 μM, in 0.015 M sodium phosphate (pH 6.6) with emission measured at 457 nm; photometer readings for this curve only were multiplied by ten before plotting. The solid line is for 1.94 μM NADH in the same buffer, plus 20 μM citrate synthase (subunit concentration), with emission measured at 428 nm. This amount of citrate synthase is enough to complex 95% of the NADH. (b) Emission spectra. Symbols and conditions as in part (a), but emission was scanned, with excitation at 340 nm.

Results

Fluorescence of NADH in the Presence of E. coli Citrate Synthase. In aqueous buffers in the neutral pH range, NADH shows a peak of fluorescence emission at 457 nm, while the excitation spectrum has a peak at 340 nm, and a smaller one at 268 nm. These spectra are well known. They both change dramatically when the NADH is bound to citrate synthase, as is shown in Figure 1. The peak of emission shifts to 428 nm, and in the excitation spectrum a new peak appears at about 285 nm in addition to that at 340 nm. Moreover, the intensity of the fluorescence is greatly increased: the fluorescence enhancement, λ (see Experimental Section), is 12.9 ± 0.3 at 428 nm. This λ value is constant over the pH range 6.6–8.0 at least, either in dilute buffers or in 0.1 M KCl. The pH independence of λ is demonstrated in Figure 3a.

These fluorescence changes are similar to those observed when NADH is bound to various dehydrogenases (see Discussion). The great increase in efficiency of light at 285 nm, in exciting the fluorescence, indicates that photons initially absorbed by protein tryptophan residues can be transferred to bound NADH by a radiationless transfer mechanism (Chen et al., 1969).

Effect of pH on NADH Binding as Measured by Fluorescence Enhancement. Inhibition of *E. coli* citrate synthase by NADH is strongest at pH values below 7.5, and falls off rapidly as the pH is raised (Weitzman, 1966b). The binding of NADH to one preparation of citrate synthase is shown in Figure 2, where data for a number of pH values are shown as Scatchard plots. The plots are apparently straight lines at all values of pH, and can be described by

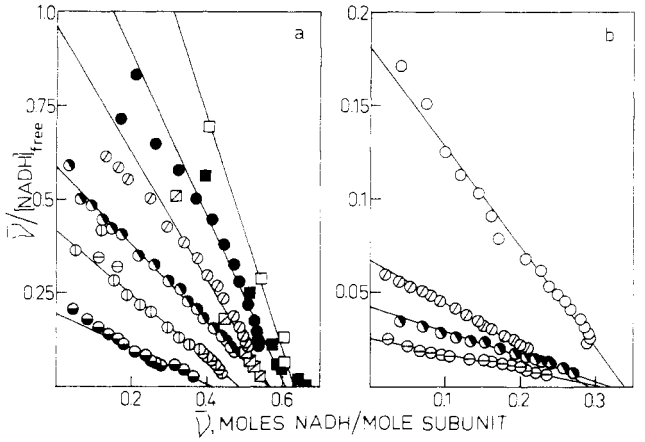

FIGURE 2: Effect of pH on NADH binding by citrate synthase, Scatchard plots. In (a) the pH values are: (□) 6.20; (● and ■) 6.65; (⊘ and ⧄) 6.93; (◒) 7.21; (◐) 7.57 (Tris-Cl buffer, 0.020 *M*); (⊖) 7.57 (phosphate buffer, 0.015 *M*); (◒) 7.81. In (b), the pH values are: (○) 7.93; (⊘) 8.17; (◔) 8.54; (⊖) 8.68. Round symbols are data for the preparation of October 1974 (final concentration, 0.222 mg/ml, 4.73 μM subunit), and square symbols for that of April 1974 (final concentration, 0.745 mg/ml, 15.8 μM subunit). Lines are drawn using parameters obtained by hyperbolic fitting of the individual curves by the method of Wilkinson (1961).

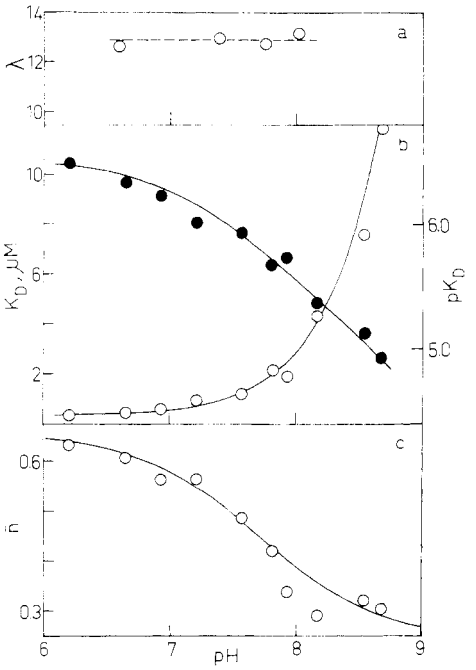

FIGURE 3: Effect of pH on NADH binding by citrate synthase, including replots of data in Figure 2. (a) pH dependence of λ, the fluorescence enhancement at 428 nm (see the text). (b) pH dependence of K_D for NADH binding (○) and of pK_D (●). The curves are calculated for K_D = 0.28 μM at low pH, and assuming that binding requires protonation of a group of pK_a = 7.05. (c) pH dependence of *n*, the number of NADH molecules bound per subunit at saturation. The line is calculated by assuming that *n* is 0.65 at low and 0.25 at high pH, and that the change in *n* involves the deprotonation of one group per subunit with pK_a = 7.7.

values of K_D (the dissociation constant for the enzyme–NADH complex) and *n* (the number of sites available per subunit of 47,000 g/mol; Tong and Duckworth, 1975). Values of K_D and *n*, from the curves in Figure 2, are plotted vs. pH in Figure 3b and c, respectively.

The effect of pH on K_D is straightforward (Figure 3b). The plot of pK_D vs. pH has a limiting slope toward the alkaline side of −1, so that the binding of NADH seems to require that one group per binding site be in the protonated form. Because the enzyme tends to precipitate at pH values below 6.5, it has been difficult to make enough measurements on the acid side of neutrality to define the binding in that range. Data from the April 1974 preparation have been added to those from the October 1974 preparation, for pH values less than 7, in constructing Figures 2 and 3. The results in Figure 3b can be approximated by assuming that K_D for NADH is 0.28 μM at low pH (with an uncertainty of perhaps 20%), and that NADH binding is dependent upon protonation of a group of pK_a approximately 7.05.

The effect of pH on K_D is enough by itself to account for the pH dependence of NADH inhibition, but the data in Figure 2 and 3 also show that the number of sites which can be occupied by NADH apparently changes from a limit of about 0.65 per subunit at low pH to about 0.25 at high pH. This transition is half-complete at pH 7.7, approximately.

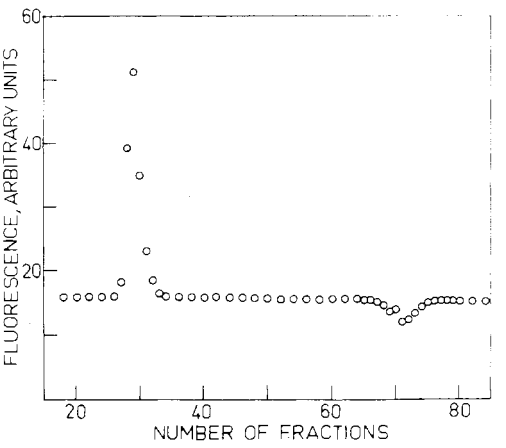

FIGURE 4: Gel filtration measurement of the binding of NADH to citrate synthase, and the enhancement of NADH fluorescence upon binding. A sample of citrate synthase, 41.3 nmol dissolved in 1.00 ml of a 0.02 *M* Tris-Cl buffer (pH 7.86) containing 18.8 μM NADH, was applied to a column of Sephadex G-50-80, 1.0 × 78.5 cm, equilibrated with the same Tris–NADH buffer. Fractions of 0.99 ml each were collected at room temperature, and the fluorescence of each was measured (excitation at 340 nm, emission at 460 nm). Fluorescence is plotted as a function of fraction number. Enzyme was found in fractions 27–33, those showing the fluorescence peak. Further explanation is given in the text. This column run gave the results presented in the third line of Table I.

Binding of NADH to Citrate Synthase Measured by Gel Filtration. The pH dependence of the apparent number of NADH binding sites just discussed is quite unusual, and we sought to verify the observation by another method. The general procedure of Hummel and Dreyer (1962) was used. Two columns of Sephadex G-50 were equilibrated with NADH at a concentration near 20 μM, at pH values of 7.00 and 7.86, respectively, and known amounts of citrate synthase, dissolved in the equilibrating solutions, were passed through the columns. Fractions of effluent were collected, and the fluorescence of each fraction was determined at 460 nm. The results of one experimental run are shown in Figure 4. All fractions fluoresce because of the present of NADH in the elution buffer. The enzyme appears at the void volume, and associated with it is a large peak of excess fluorescence due to NADH whose fluorescence is enhanced because it is bound to the enzyme. Somewhat later in the elution profile appears a trough, a deficiency of NADH corresponding to the amount of coenzyme bound to the citrate synthase. The area of the trough measures the amount of NADH bound, and the area of the

Table I: Measurements of NADH Binding to Citrate Synthase, and the Fluorescence Enhancement Ratio at 460 nm, by Gel Filtration.[e]

Conditions of Measurements	Enzyme Subunits Added (nmol)	Area of Peak,[a] Apparent nmol of NADH	Area of trough,[a] Apparent nmol of NADH	λ_{460}[b]	NADH[c] Bound, from Area of Trough (nmol/nmol)	NADH[d] Bound, Predicted from Data in Figure 3 (nmol/nmol)
0.015 *M* Sodium phosphate (pH 7.00); 21.1 μM NADH	37.3	125 ± 2	21.5 ± 2.2	5.8 ± 0.6	0.58 ± 0.06	0.55
	37.3	117 ± 2	20.9 ± 2.3	5.6 ± 0.6	0.56 ± 0.06	0.55
0.02 *M* Tris-Cl (pH 7.86); 18.8 μM NADH	41.3	107 ± 2	15.6 ± 2.0	6.9 ± 0.9	0.38 ± 0.05	0.37
	37.2	96 ± 2	16.2 ± 2.2	5.9 ± 0.8	0.44 ± 0.06	0.37

[a] Errors in areas were based on an estimated uncertainty of ±0.13 μM in apparent NADH concentrations in all fractions. [b] λ_{460} = (area of peak)/(area of trough). [c] NADH bound = (area of trough)/(enzyme subunits added). [d] These predictions were made from the values of n and K_D read from Figure 3, for pH 7.00 or 7.86, using 21.1 μM free NADH at pH 7.00 and 18.8 μM at pH 7.86. [e] All measurements were performed at room temperature. The column used for the runs at pH 7.00 was 1.0 × 80 cm, and that for runs at pH 7.86 was 1.0 × 78.5 cm. The run illustrated in Figure 4 is that in the *third* line of this table. For further explanation see the text.

peak divided by the area of the trough is λ, the fluorescence enhancement due to binding. Because all of the fluorescence measurements in this experiment were at 460 nm, rather than 428 nm, the value of λ is not 12.9, but by consideration of Figure 1b can be predicted to be about 5.8. The results of four gel filtration binding runs—two each at pH 7.00 and pH 7.86—are presented in Table I.

The fact that the fluorescence enhancement method indicated considerably less than one tight NADH binding site per subunit (Figures 2 and 3) immediately suggested the possibility that not all NADH molecules are bound in a fluorescent form, or at least that for some bound molecules the fluorescence is not strongly enhanced. If this were the case, then the enhancement factor measured by the peak-to-trough ratio in the gel filtration experiments would be less than the predicted value of about 5.8, since such molecules would contribute more to the trough than the peak. Four separate measurements of this ratio are given in Table I, and all are in the range 5.6–6.9, with estimated uncertainties of about 15%, essentially the same as the predicted value. This result must mean that there are no "invisible" bound NADH molecules in this concentration range, which can bind to citrate synthase but go undetected by the fluorescence enhancement method. The last two columns of Table I provide further confirmation of this conclusion. They show that the amount of NADH binding measured by the gel filtration method at both pH values agrees well, within the errors, with that calculated from the binding parameters we measured by fluorescence enhancement.

The experiments described in the following sections were performed at or near pH 7.8. This is in the midst of the pH-dependent transitions just described, but it has two advantages. First, the binding of NADH is weak enough at this pH that it can be affected readily by various antagonists. Second, the enzyme activity is usually assayed at pH 7.8, where it is substantial, though it decreases rapidly toward the acid side (Weitzman, 1966b; Faloona and Srere, 1969); thus binding measurements at pH 7.8 may be directly compared with various kinetic observations in the literature. Almost the same pH value, 8.0, was chosen for equilibrium dialysis studies on this enzyme by Wright and Sanwal (1971).

Table II: Affinity of Citrate Synthase for Various Nucleotides at pH 7.8.

Nucleotide	K_D or K_i[a] (μM)	Nucleotide	K_D or K_i[a] (μM)
NADH	1.6 ± 0.1	3′-AMP	65 ± 4
NADPH	53 ± 5[b]	NAD^+	1100 ± 200
5′-AMP	83 ± 5	$NADP^+$	1900 ± 300

[a] K_D values for NADH and NADPH were from fluorescence titrations; K_i values for the other nucleotides were determined from their abilities to act as competitive inhibitors of NADH binding. [b] Assuming 0.42 site for NADPH per subunit at pH 7.8, the number found for NADH.

Specificity of the NADH Binding Sites. A number of compounds structurally related to NADH were tested for their ability to compete with NADH for its binding sites on citrate synthase. Some competition was seen in all cases. The effect of the compounds was to increase K_D for NADH without changing the number of sites at saturation, and the effect on K_D was linear with concentration, and given by

$$K_D^{obsd} = K_D(1 + [I]/K_i)$$

where [I] is the concentration of the competing compound, and K_i is its apparent binding constant. Values of K_i for the different compounds tried are presented in Table II. As can be seen, NAD^+ and $NADP^+$ were only weakly competitive, while stronger inhibition was seen with both 3′- and 5′-AMP. Weitzman (1967) has shown that 5′-AMP does not inhibit *E. coli* citrate synthase, but that it will reverse NADH inhibition. The effect of 3′-AMP on enzyme activity will be dealt with below.

Because NADPH is itself fluorescent, its binding to citrate synthase could be measured directly by the method being used for NADH binding. One titration of a small amount of NADPH with large amounts of enzyme was performed (data not shown), and this gave λ = 14 ± 1.5 at 428 nm for fully complexed NADPH, and K_D = 53 ± 10 μM, assuming the same number of sites for NADPH, 0.42 per subunit at this pH, as for NADH. In this experiment, a maximum of 23% of the NADPH was complexed, so that

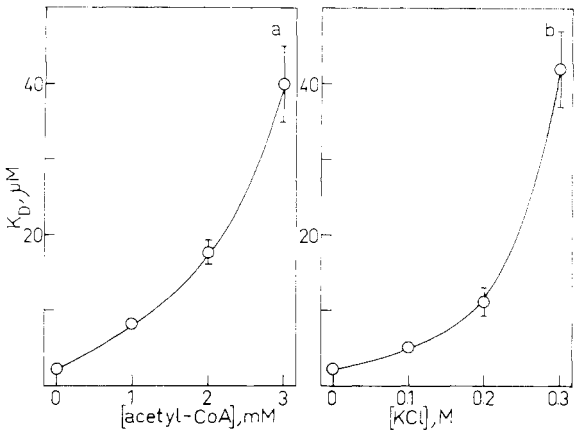

FIGURE 5: Effects of acetyl-CoA and KCl on K_D for the NADH–citrate synthase complex. Measurements were performed with 0.222 mg/ml of citrate synthase (4.73 μM subunit), preparation of October 1974, in 0.02 M Tris-Cl (pH 7.8). (a) Effect of acetyl-CoA; (b) effect of KCl. The curves were drawn by eye, and have no special significance.

the extrapolation to give λ was long. Even at this low a degree of complexation, however, the maximum of fluorescence emission for the NADPH had shifted from 457 to 437 nm, and so it is possible that NADPH *fully* complexed to citrate synthase would be found to have the same fluorescence emission spectrum, with a maximum at 428 nm, as does NADH bound to the enzyme.

Effects of Substrates and Other Effectors on NADH Binding. Acetyl-CoA, one of the substrates of citrate synthase, weakens the binding of NADH without changing the number of sites. A plot of K_D^{obsd} vs. [acetyl-CoA] is shown in Figure 5a. Since NADH inhibition is mainly competitive with acetyl-CoA (Weitzman, 1966a; Wright and Sanwal, 1971), this result is expected from the kinetics. NADH binding is also weakened by KCl (Figure 5b), which is known to activate the *E. coli* enzyme (Faloona and Srere, 1969) and abolish NADH inhibition (Weitzman, 1966b). It will be noted that the curves in Figure 5a and b are concave up, suggesting cooperativity in acetyl-CoA and KCl binding. The concentration of KCl needed to raise the K_D^{obsd} for NADH to twice the control value is 0.09 ± 0.01 M, while that for acetyl-CoA is 0.45 ± 0.05 mM; these values presumably represent the half-saturation values for binding of KCl and acetyl-CoA to their respective sites on citrate synthase. Direct measurements of the binding of acetyl-CoA to citrate synthase were performed by Wright and Sanwal (1971), and they obtained a slightly cooperative saturation curve, indicating at least 0.9 site per 61000 g of protein (at least 0.7 site per subunit) and a half-saturation value of 0.4–0.6 mM at pH 8.0.

It is of interest that 0.2 M KCl, which completely abolishes NADH inhibition in standard assays (Weitzman, 1966b), still permits significant binding of NADH, with a K_D^{obsd} of about 11 μM (Figure 5b). This fact raises the possibility that the effect of KCl is stronger when one or more substrates are present. We have found that 0.1 M KCl and 2 mM acetyl-CoA, present together, abolish all enhancement of NADH fluorescence by citrate synthase, at least in the range of concentrations used in the studies described in this paper (data not shown). KCl and acetyl-CoA therefore can act synergistically, together preventing NADH binding much more effectively than their separate effects would predict. (The value of K_D^{obsd} predicted from the curves in Figure 5, for these levels of KCl and acetyl-CoA, may be calculated to be about 21 μM; this value would have led to a very noticeable fluorescence enhancement.)

α-Ketoglutarate, an inhibitor of *E. coli* citrate synthase whose mode of action is believed to be allosteric (Wright et al., 1967), has no effect on NADH binding at pH 7.8, at a concentration of 0.99 mM, although it is strongly inhibitory in standard assays at this level. This compound also made no difference to the weakening of NADH binding observed in the presence of 2 mM acetyl-CoA or 0.1 M KCl. The other substrate, oxaloacetate, had little or no effect on NADH binding at concentrations as high as 0.1 mM. These data are not shown here.

Effect of 3′-AMP on Enzyme Activity. Since 3′-AMP is an analogue of acetyl-CoA (corresponding to the adenylate portion of the coenzyme), it might be expected to bind in the active site of citrate synthase, competing with acetyl-CoA. As we noted above, 3′-AMP does inhibit *NADH* binding with a K_i of 65 μM (Table II) and since acetyl-CoA also inhibits NADH binding, the effect of 3′-AMP might be a result of its occupation of the acetyl-CoA sites. We could test for this possibility kinetically, by seeing whether 3′-AMP inhibits citrate synthase. At pH 7.8, we found that it is an inhibitor which is competitive with acetyl-CoA (K_{is} = 0.34 mM) and noncompetitive with oxaloacetate ($K_{is} = K_{ii}$ = 0.17 mM). (The terminology of Cleland (1963) is used.) These experiments were done in the presence of 0.1 M KCl, since substrate saturation curves are hyperbolic in this medium, whereas that for acetyl-CoA is quite sigmoid in the absence of KCl (Faloona and Srere, 1969; Wright and Sanwal, 1971), and analysis of inhibition patterns is difficult. Inhibition by 3′-AMP does still occur, however, when KCl is not present (data not shown).

Quenching of Protein Fluorescence by Bound NADH. *E. coli* citrate synthase displays an intrinsic fluorescence centered at 343 nm whose peak of excitation is at 287 nm (spectra not shown). This fluorescence of course is to be attributed to tryptophan, of which each citrate synthase subunit contains four residues (Tong and Duckworth, 1975). Since the absorption peak of NADH largely overlaps the protein fluorescence emission envelope, radiationless transfer of photons from excited tryptophan chromophores to NADH molecules is possible if the NADH molecules are close enough to the tryptophans, and in suitable orientations to them (Chen et al., 1969). The substantial increase in efficiency of light at 285 nm in exciting fluorescence of NADH, when the coenzyme is bound to citrate synthase (see Figure 1), shows that this transfer of energy occurs. It can also be measured readily by the degree of quenching of tryptophan fluorescence as a function of amount of NADH bound to the enzyme.

In Figure 6 we show sample plots of $(F_o - F_{\bar{\nu}})/F_o$ vs. $\bar{\nu}$, where F_o and $F_{\bar{\nu}}$ are the intensities of protein fluorescence at 343 nm when NADH is absent, and bound to the enzyme at ν molecules per subunit, respectively. Thus $(F_o - F_{\bar{\nu}})/F_o$ is the fraction of protein fluorescence quenched when $\bar{\nu}$ molecules of NADH are bound. (In making these measurements it is necessary to correct for the "trivial" absorption of fluorescent light by unbound NADH present in the solution, and also for partial absorption of excitation light at 287 nm by added NADH.) The plots of $(F_o - F_{\bar{\nu}})/F_o$ vs. $\bar{\nu}$ appear to be straight lines, or very nearly so, and as expected they go through the origin. The best line through all the data has a slope of 1.06 ± 0.10, which means that when half the subunits in one molecule of citrate synthase have bound

molecules of NADH, 53 ± 5% of the tryptophan fluorescence is quenched. Thus all of the tryptophan residues in the enzyme are close enough to the NADH sites to permit radiationless transfer of photons from them to the nicotinamide chromophores.

Discussion

Boyer and Theorell (1956) first studied the enhancement of NADH fluorescence when the coenzyme is bound to horse liver alcohol dehydrogenase, and many other dehydrogenases are known to have the same effect (see, e.g., Velick, 1958; Dickinson, 1970; von Ellenrieder et al., 1972; Harvey et al., 1972). The present communication describes what may be the first example of this phenomenon in which NADH plays a strictly regulatory role. As with the dehydrogenases, citrate synthase causes an increase in NADH fluorescence yield, with a shift of the emission spectrum toward the blue. The excitation maximum remains at 340 nm, but a new peak appears at 285–290 nm (Figure 1). This latter peak is due to primary absorption of light by protein tryptophans, which then transfer the energy to NADH. This transfer also leads to a quenching of tryptophan fluorescence by bound NADH (Figure 6).

We have no certain explanation of the discrepancy between our results and those of Wright and Sanwal (1971), who found very little NADH binding by equilibrium dialysis at pH 8.0. Their measurements were performed at 4°C, with a protein concentration of 14.6 mg/ml (Wright and Sanwal, 1971), however, while ours were at 21 ± 1°C with a protein concentration of 0.13–1.3 mg/ml; and it may be that temperature, or protein concentration, or both, have a large effect on NADH binding. Specific experiments will be needed to test these possibilities.

The pH dependence of K_D for NADH binding (Figures 2 and 3) suggests that the binding requires the protonation of an acid-dissociable group of pK_a about 7.05. This pH dependence accounts for the disappearance of NADH inhibition at high pH, an effect discovered by Weitzman (1966b). In addition to its effect on K_D, raising the pH reduces the number of observed NADH binding sites from about 0.65 to about 0.25 per subunit; the midpoint of the change lies at about pH 7.7. Since the subunits of this enzyme are apparently identical (see Tong and Duckworth, 1975, and references therein), one would customarily expect one site for each subunit, at all values of pH. The enzyme is known to depolymerize considerably over the pH range 7–9, however (Wright and Sanwal, 1971; Tong and Duckworth, 1975), and it is quite possible that the different polymeric forms have different affinities for NADH. At pH 7 there is a mixture of oligomers, ranging from monomers to decamers or greater, while at pH 9, at least at protein concentrations less than 1 mg/ml, only dimer is seen (Tong and Duckworth, 1975). The change in the number of NADH sites which occurs between pH 6.20 and pH 8.68 may be one reflection of the marked changes in the quaternary structure of citrate synthase which occur in the same pH range.

To explain the effects of various inhibitors of NADH binding we postulate two types of binding sites on the enzyme: the *active sites* which bind acetyl-CoA and its competitive inhibitor, 3′-AMP; and the *allosteric sites* which bind NADH and probably NADPH, and have a slight affinity for NAD^+ and $NADP^+$. Active sites and allosteric sites, in this view, cannot be occupied at the same time, and so acetyl-CoA is pictured as including a conformational change which destroys the allosteric sites. The effect of KCl

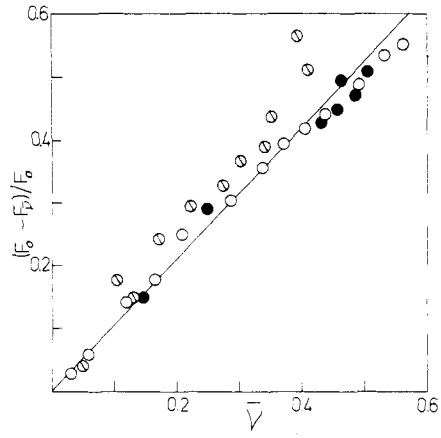

FIGURE 6: Quenching of intrinsic fluorescence of citrate synthase by bound NADH. The fraction of fluorescence quenched, $(F_o - F_{\bar{\nu}})/F_o$, is plotted against $\bar{\nu}$, the number of molecules of NADH bound per subunit. Excitation was at 285 nm, and emission at 343 nm; enzyme was 0.745 mg/ml of the April 1974 preparation (15.8 μM subunit), in 0.02 M Tris-Cl buffers. Three data sets, out of eight similar ones, are shown. The points are (●) pH 6.6; (○) pH 7.8; (⊘) pH 7.8, plus 1.0 mM acetyl-CoA. The line is straight, has a slope of 1.06, and passes through the origin. See the text for its significance.

may be to promote this same change, simultaneously abolishing NADH binding and stimulating both acetyl-CoA binding and enzyme activity. NADH inhibits the enzyme presumably because it prevents this conformational change.

The other nucleotide studied, 5′-AMP, cannot be binding to the active sites, since it is not an inhibitor of the enzyme. Its similarity in structure to one-half of the NADH molecule suggests that it might occupy part of the allosteric site; if this is the case, then it must be the nicotinamide part of the coenzyme that is involved in any allosteric conformational change. Alternatively, 5′-AMP may bind to still another class of site, bringing on a conformational change like that induced by acetyl-CoA. This explanation requires the *ad hoc* postulation of a further class of allosteric sites, and so may be less attractive.

Since the other apparently allosteric inhibitor, α-ketoglutarate, does not affect NADH binding, at least at pH 7.8, it seems not to act by inducing the same conformational change that NADH induces. The critical observations bearing on this question are those made when acetyl-CoA and α-ketoglutarate, or KCl and α-ketoglutarate, were present together in an NADH binding experiment. In both cases, the binding curves were the same as if no α-ketoglutarate were present, indicating that α-ketoglutarate cannot oppose the conformational change which acetyl-CoA and KCl are believed to induce.

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The Structure of the Covalent Adduct Formed by the Interaction of 3-Dimethylamino-1-propyne and the Flavine of Mitochondrial Amine Oxidase†

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ABSTRACT: 3-Dimethylamino-1-propyne irreversibly inactivates mitochondrial monoamine oxidase from bovine liver. The inactivation results in the loss of absorption in the 450–500-nm region of the flavine spectrum and a concomitant increase in absorbance at 410 nm. For the enzyme-bound adduct ϵ_{410} = 28000. The spectral properties of the adduct of the liver enzyme with 3-dimethylamino-1-propyne are similar to those observed when the pig kidney enzyme is inactivated with pargyline (Chuang et al. (1974), *J. Biol. Chem. 249,* 2381). From a proteolytic digest of the enzyme inactivated with labeled inhibitor a flavine peptide has been isolated which contains 1 mol of inactivator/mol of flavine. The chemical and spectral properties of the adduct are those of compounds containing the structure —N—CH=CH—CH=N⁺<. It was concluded that the flavine–inhibitor adduct is a N-5 substituted dihydroflavine and its structure has been determined.

Monoamine oxidase (MAO)[1] [EC 1.4.3.4], an enzyme containing covalently bound FAD (Nara et al., 1966; Erwin and Hellerman, 1967; Kearney et al., 1971), has been a favorite inhibitor target since 1952 when the inhibition of its activity was correlated with antidepressant activity in patients following the administration of iproniazid (Selikoff et al., 1952). Literally hundreds of inhibitors have been described (Ho, 1972), many of which have been used in vivo to help elucidate the physiological and psychological roles of the neurohormones. Several of these compounds have been used clinically as either antidepressive or antihypertensive agents. Little is known, however, about the mechanism of action of these inhibitors.

Pargyline, an acetylenic amine, has been more extensively investigated than other inhibitors. In 1968 it was shown that inhibition of bovine kidney MAO by pargyline is irreversible (Hellerman and Erwin, 1968), but can be protected against by benzylamine; that the inhibitor combines with the oxidized form of the enzyme; and that inhibition results in the formation of a 1:1 covalent adduct between the enzyme and the inhibitor. Inactivation of MAO from pig liver mitochondria by pargyline has been studied by Oreland et al. (1973). Recently, Chuang et al. (1974) reported that the inhibition of kidney MAO by pargyline is accompanied by disappearance of the 455-nm band of the flavoquinone and the appearance of a new band at 410 nm. This fact and the observation that [7-^{14}C]pargyline is recovered in the flavine peptide fraction after proteolytic digestion were taken as ev-

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[1] Abbreviations used are: MAO, monoamine oxidase; pargyline, *N*-methyl-*N*-benzyl-2-propynylamine; [^{14}C]CH$_2$ inactivator, 3-dimethylamino[3-^{14}C]-1-propyne; [^{14}C]CH$_3$ inactivator, 3-(*N*,*N*-[^{14}C]dimethylamino)-1-propyne.