enzyme they act upon. For PATH, the situation is exactly the opposite (the inhibitor TPN^+ is a potential substrate) and can be explained by the particular metabolic role of the enzyme (see Figure 3). Unlike the above-mentioned regulatory enzymes, PATH is not the first catalyst of a multistage metabolic pathway, but the essential link between two pathways. Since the enzyme has to comply with the physiological necessity of unidirectional catalysis, the direct control by the product TPN^+ appears to be the best way of regulation (if not the only possible one). The fact that TPN^+ behaves as negative effector explains the irreversibility of TPN^+ evolution, but is also responsible for the TPN^+ inhibition of the TPNH–DPN^+ reaction. The latter effect could correspond to a kind of "metabolic buffer effect" averting too large a consumption of TPNH, which is also needed for biosynthetic purposes.

References

Cohen, P. T. (1967), Ph.D. Thesis, Brandeis University, Waltham, Mass., Ann Arbor, Mich., University Microfilms Inc., No. 67-16542.

Cohen, P. T., and Kaplan, N. O. (1970a), *J. Biol. Chem. 245,* 2825-2836.

Cohen, P. T., and Kaplan, N. O. (1970b), *J. Biol. Chem. 245,* 4666-4672.

Cohen, R. (1963), *C. R. Hebd. Seances Acad. Sci., Ser. C 256,* 3513-3515.

Colowick, S. P., Kaplan, N. O., Neufeld, E. F., and Ciotti, M. M. (1952), *J. Biol. Chem. 195,* 95-105.

Doelle, H. W. (1969), In Bacterial Metabolism, New York, N.Y., Academic Press, 352-401.

Fewson, A., and Nicholas, D. J. D. (1961), *Biochim. Biophys. Acta 49,* 335-349.

Hanson, K. R. (1966), *J. Mol. Biol. 22,* 405-409.

Haschemeyer, R. H., and de Harven, E. (1974), *Annu. Rev. Biochem. 43,* 279-301.

Horton, H. R., Swaisgood, H. E., and Mosbach, K. (1974), *Biochem. Biophys. Res. Commun. 61,* 1118-1124.

Kemper, D., and Everse, J. (1973), *Methods Enzymol. 27,* 67-82.

Louie, D. D., Kaplan, N. O., and Mc Lean, J. D. (1972), *J. Mol. Biol. 70,* 651-664.

Monod, J., Changeux, J. P., and Jacob, F. (1963), *J. Mol. Biol. 6,* 306-329.

Monod, J., Wyman, J., and Changeux, J. P. (1965), *J. Mol. Biol. 12,* 88-118.

Ochoa, S. (1955), *Methods Enzymol. 1,* 699-704.

Stern, I. J., Wang, C. H., and Gilmour, C. M. (1960), *J. Bacteriol. 79,* 601-611.

Widmer, F., and Kaplan, N. O. (1976), *Biochemistry 15,* (preceding paper in this issue).

Coenzyme Binding by Triphosphopyridine Nucleotide Dependent Isocitrate Dehydrogenase from Beef Liver. Equilibrium and Kinetics Studies†

Marie France Carlier* and Dominique Pantaloni

ABSTRACT: The binding of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to nicotinamide adenine dinucleotide phosphate (NADP) dependent isocitrate dehydrogenase from beef liver cytoplasm was studied by several equilibrium techniques (ultracentrifugation, molecular sieving, ultrafiltration, fluorescence). Two binding sites (per dimeric enzyme molecule) were found with slightly different dissociation constants (0.5 and 0.12 μM) and fluorescence yields (7.7 and 6.3). A ternary complex was formed between enzyme, isocitrate, and NADPH, in which NADPH dissociation constant was 5 μM. On the contrary, no binding of NADPH to the enzyme took place in the presence of magnesium isocitrate. Dialysis experiments showed the existence of 1 NADP binding site/dimer, with a dissociation constant of 26 μM. When NADPH was present with the enzyme in the proportion of 1 molecule/dimer, the dissociation constant of NADP was decreased fourfold, reaching a value quantitatively comparable to the Michaelis constant. The kinetics of coenzyme binding was followed using the stopped-flow technique with fluorescence detection. NADPH binding to the enzyme occurred through one fast reaction (k_1 = 20 μM^{-1} s^{-1}). Dissociation of NADPH took place upon NADP binding; however, equilibrium as well as kinetic data were incompatible with a simple competition scheme. Dissociation of NADPH from the enzyme upon magnesium isocitrate binding was preceded by the formation of a transitory ternary complex in which the fluorescence of NADPH was only about 30% of that in the enzyme–NADPH complex. The interaction between the coenzymes and the involvement of ternary complexes in the catalytic mechanism are discussed in relation with what is known about the regulatory role of the coenzyme (Carlier, M. F., and Pantaloni, D. (1976), *Biochemistry 15,* 1761-1766).

Previous studies (Carlier and Pantaloni, 1973) have shown that isocitrate dehydrogenase (*threo*-D_s-isocitrate:$NADP^+$ [1] oxidoreductase (decarboxylation) EC 1.1.1.42) purified from beef liver cytoplasm is a dimeric enzyme of molecular weight 48 000 × 2. In the absence of divalent metal cations, steady-state kinetics exhibit catalytic activation by NADPH, the reaction product (Carlier and Pantaloni, 1976a). It has been demonstrated that NADPH did not play a redox role in this activation and was probably involved in the second step (de-

† From the Laboratoire d'Enzymologie du C.N.R.S., 91190 Gif-sur-Yvette, France. *Received May 17, 1976.*

Abbreviations used are: NAD, nicotinamide adenine dinucleotide; NADP, NAD phosphate; NADPH, reduced NADP; EDTA, (ethylenedinitrilo)tetraacetic acid.

carboxylation) of the reaction (Carlier et al., 1976). NADP was competitive towards NADPH in this activation. Under certain conditions, the activity of the enzyme is regulated by the ratio of NADPH to NADP concentrations. Other authors have shown that NADPH was necessary in the enzymatic catalysis of β-hydrogen exchange of 2-oxoglutarate, which is the presumed first step of the reverse reaction of isocitrate dehydrogenase (Rose, 1960). Again, NADPH does not have a redox role in this reaction (Rippa et al., 1974). Different models have been proposed which could account for these kinetic results, but a direct study of coenzyme interaction with the protein was necessary to determine more precisely the different roles of the coenzyme in the catalytic mechanism. With this purpose in mind, we performed equilibrium experiments, such as ultrafiltration, dialysis, analytical ultracentrifugation, fluorescence titrations, and stopped-flow binding kinetics experiments. Our data are discussed and compared with those obtained recently by Ehrlich and Colman (1975) on the mitochondrial pig heart enzyme.

Materials and Methods

Materials. Beef liver cytoplasmic isocitrate dehydrogenase ($NADP^+$) was purified as previously described (Carlier and Pantaloni, 1973). Protein concentration was determined using a Beckman Acta V recording spectrophotometer, and assuming a value of 1.29 cm^{-1} for the extinction coefficient ϵ 278 (0.1%). Enzyme concentrations were calculated in terms of protomer units of molecular weight 48 000. The salts used in the buffers were Merck analytical grade. We used 0.1 M sodium phosphate buffer, pH 7.4, named "buffer P". All solutions were treated with Chelex-100 resin (Bio-Rad) when it was necessary to have no divalent metal cations in the solutions. DL-Isocitrate trisodium salt, NADP, and NAPDH were purchased from Sigma. Coenzyme concentrations were determined spectrophotometrically. The carbonyl-labeled [^{14}-C]NADP (45 mCi/mmol) was obtained from Amersham.

Gel Filtration Binding Studies. The number of NADPH binding sites was determined by filtration through Sephadex G-25 according to Hummel and Dreyer (1962). A 0.7 × 30 cm column was poured with Sephadex G-25 (coarse) and equilibrated with buffer P containing 11.7 μM NADPH. The same buffer was pushed through the column by a Gilson peristaltic pump, at a flow rate of 0.34 ml/min. Twenty microliters of an enzyme solution at 6.98 mg/ml were applied onto the top of the column at time zero. The absorbance of the eluted solution was continuously recorded at 340 nm using a Cary 15 spectrophotometer equipped with a flow cell. The elution pattern showed a peak, followed by a trough. The area of the peak was equal to that of the trough and represented the quantity of NADPH bound to the enzyme. The number of sites was given by the ratio of number of bound coenzyme molecules to the number of enzyme molecules deposited on the column.

Analytical Ultracentrifugation Measurements. This hydrodynamic method (Steinberg and Schachman, 1968) is similar to the preceeding one in its principle. Free NADPH was separated from the enzyme and enzyme–coenzyme complex by sedimentation. The analysis of the variation of absorbance, at 340 nm, from the meniscus to the bottom of the cell, was performed from photographs of the cell taken at different times during the sedimentation. This method leads to the determination of free and bound NADPH concentrations, number of binding sites, and sedimentation coefficient of the enzyme–NADPH complex. Scanning of the photographs was performed with a Joyce-Loebl densitometer.

Fluorescence Measurements. Fluorescent titrations were performed at 20 °C with an Aminco-Bowman spectrofluorimeter equipped with a thermostated cell block. Excitation and emission slit widths were varied, according to the enzyme concentration used, between 0.5 and 3 mm. All spectra and equilibrium measurements were recorded on a XY Climatic M100 recorder. Fluorescent titrations of the enzyme by NADPH were carried out in a cell containing 1.5 ml of buffer solution and a known enzyme concentration. Concentrated NADPH was added to the cell in 2–20-μl aliquots. No more than 100 μl was added to the cell. To correct for dilution and inner-filter effects, the same experiment was carried out in the same cell containing the same volume of buffer. The range of enzyme concentrations was 0.1–20 μM, and that for NADPH was 0–30 μM. Alternatively, continuous automatic titrations were performed with an Agla microsyringe whose pestle was pushed mechanically so that NADPH was delivered to the cell with a controlled flow rate through a Teflon capillary. The cell content was magnetically stirred during the experiment.

Ultrafiltration Binding Experiments. The binding of NADPH to isocitrate dehydrogenase was studied by ultrafiltration using Diaflo PM10 membranes (25-mm diameter) in an Amicon 10 PA ultrafiltration apparatus. A solution (2.5 ml) containing known concentrations of enzyme and NADPH was placed in the cell and a pressure of 4.5 bars was applied. The first 50 μl was discarded, and the following 200 μl was used to measure the concentration of free NADPH with a fluorescence assay (excitation 340 nm, emission 460 nm). No passage of the enzyme through the membrane was detected from catalytic activity.

Equilibrium Dialysis. Binding experiments were conducted with [^{14}C]NADP using altuglas cells kindly lent by Dr Kerjan. Each compartment of 5-mm inner diameter contained 50 μl. Cells are equipped with Rhone Poulenc IRIS 3069 membranes which allow the equilibrium concentration of nucleotides to be reached within 3 h. The cells were filled up with a Hamilton microsyringe and mechanically shaken at 4 °C, usually overnight and only for 5 h when NADPH was present. Under both conditions, integrity of the enzyme activity was checked at the end of the experiment. A 20-μl aliquot was taken out of each compartment and deposited on a Whatman GF/C filter. Filters were dried and radioactivity was measured in Bray's scintillation fluid (Bray, 1960) using a Packard Tri-Carb liquid scintillation counter.

Kinetic Experiments. The kinetics of binding of coenzymes to isocitrate dehydrogenase was followed with a Durrum Gibson stopped-flow apparatus using fluorescence detection. The light was provided by a 450-W Osram xenon lamp, and the excitation wavelength was selected by a grating. It was emitted with 6-nm slit width, and was prevented from impinging upon the photomultiplier by a Corning CS n° 3.72 filter placed on the emission side of the cell. Variations in fluorescence intensity were recorded on a Tektronix storage oscilloscope. Under the pressure conditions used (6 kg/cm^2), the dead time of the apparatus, determined from the pseudo-first-order reaction of ferricyanure with ascorbate, was 1.8 ms. Experiments were performed at 20 °C in buffer P. The enzyme concentration, after mixing of the reactants, was usually 7–10 μM.

Results

Number of Binding Sites for NADPH. By the Sephadex G-25 molecular sieving method, 0.9 ± 0.1 site/protomer was found when the eluting solution was buffer P + NADPH. When the substrate magnesium isocitrate, at a saturating concentration, was added to the eluting buffer, no peak ap-

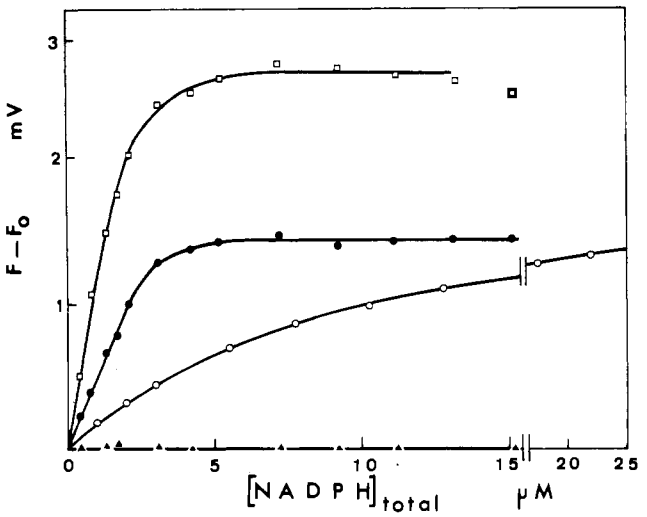

FIGURE 1: Coenzyme fluorescence titrations of the enzyme with NADPH. The cell contained buffer P and 3.8 μM enzyme subunits, with the following additions: (●) none; (○) 20 mM DL-isocitrate (previously treated by chelex-100 resin); (▲) 3.8 mM DL-isocitrate and 7.7 mM $MgSO_4$; (□) 15.4 mM DL-isocitrate and 7.7 mM EDTA. Correction for dilution and inner-filter effects was assured by subtraction of free NADPH fluorescence, which was measured in a separate experiment.

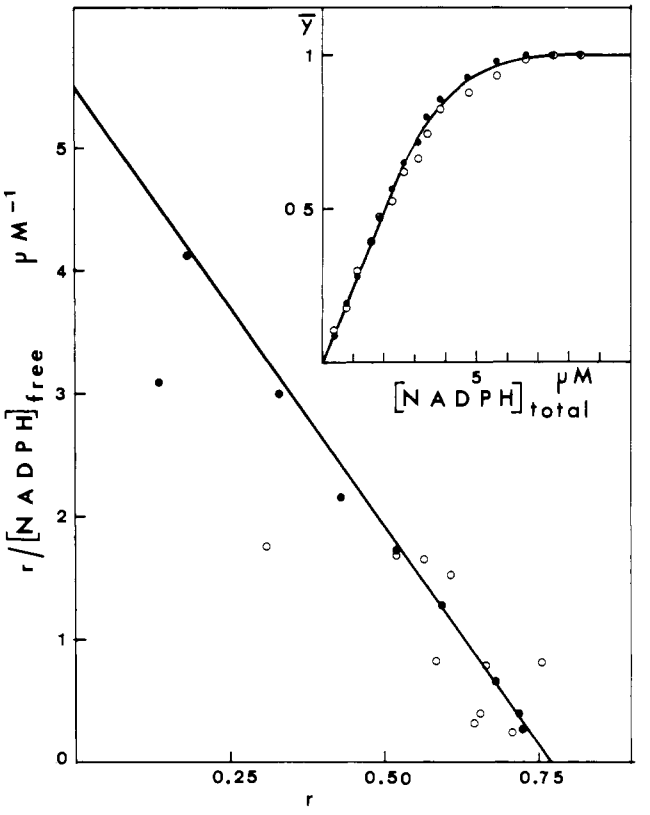

FIGURE 2: Binding of NADPH to isocitrate dehydrogenase. Scatchard plots: mol of NADPH bound per mol of enzyme protomer divided by the free NADPH concentration, r/NADPH vs. r, at an enzyme concentration of 3.98 μM in buffer P. (●) Data obtained from a coenzyme fluorescence titration; (○) data obtained from direct binding measurements using ultrafiltration. Inset: binding of NADPH plotted against total NADPH concentration, followed by NADPH fluorescence monitoring (●) and protein fluorescence quenching (○). The enzyme concentration was 5.4 μM.

peared, indicating that NADPH does not bind to the complex enzyme-magnesium isocitrate. The same number of binding sites was obtained from analytical ultracentrifugation experiments. The sedimentation coefficient of the complex E-NADPH was 5.6 S at the concentration of 3 mg/ml, a value close to that of 5.76 S found for the native enzyme at the same concentration (Carlier and Pantaloni, 1973).

Dissociation Constant of NADPH. When the enzyme, in the concentration range of 1–5 μM, was titrated by NADPH, the binding of NADPH was accompanied by a blue shift of the fluorescence emission wavelength of the coenzyme from 460 to 430 nm, and an enhancement of the fluorescence intensity by a factor of about 6.5 at 430 nm. The excitation wavelength was not appreciably shifted ($\Delta\lambda < 5$ nm). Otherwise, we did not observe any appreciable absorption difference spectrum, in the region of 340 nm, between bound coenzyme and free coenzyme. When the enzyme was at a concentration of 1–5 μM, saturation of the enzyme by NADPH showed a very high affinity of the coenzyme and a titration curve was obtained. Experimental plots could be fit to a stoichiometric straight line, indicating 0.7–0.8 binding site per protomer subunit of molecular weight 48 000 (Figure 1). In another experiment, a known amount of NADPH was titrated by the enzyme. Since the binding of NADPH to the enzyme is tight, the assumption can be made that when the enzyme concentration is much lower than NADPH concentration, the only enzyme species present in solution is $E(NADPH)_2$. The fluorescence emission spectrum of NADPH in this complex appeared homothetic to that of NADPH in the E-NADPH complex, which is supposed to be the predominant fluorescent species when the enzyme was in great excess with respect to NADPH. So, in later experiments, all fluorescence measurements were carried out at the emission wavelength of 430 nm. It has been shown that cytoplasmic isocitrate dehydrogenase undergoes a monomer-dimer reaction with an equilibrium association constant of approximately 10^7 M^{-1} (Carlier and Pantaloni, 1973). Under the conditions of protein concentration >1 μM, the dimeric species is predominant. Due to the high affinity of NADPH for the enzyme, fluorescence measurements performed in the 1–5 μM enzyme concentration range are useful for the determination of the number of binding sites and fluorescence enhancement, but inadequate for the estimation of dissociation constants. However, if a linear relation is assumed between the fluorescence intensity and the number of bound NADPH molecules, in the region of 70–95% saturation, these data can be fitted to a Michaelian isotherm and are in good correlation with the results of ultrafiltration experiments. The Scatchard representation (Scatchard, 1957) shows that 0.75 site/monomer is titrated with a single dissociation constant of 0.15 ± 0.03 μM (Figure 2). A similar correlation between fluorescence and ultrafiltration data was obtained when the analysis was carried out according to Klotz (1946) and when the "difference method" of Kurganov applied to ligand fluorescence titrations was used (Kurganov et al., 1972; Silverberg and Dalziel, 1974).

NADPH binding was also accompanied by a 26% quenching of protein fluorescence at 335 nm when tryptophan residues were excited at 290 nm. The enzyme has been shown to contain 7 tryptophan residues/protomer (unpublished results). Actually, energy transfer takes place from excited tryptophans to the nicotinamide ring of NADPH. The probability of transfer was calculated to be 8%. Binding results derived from protein fluorescence quenching measurements were also in good agreement with coenzyme fluorescence titrations (Figure 2). All these experiments give reasonable support to the assumption that fluorescence enhancements accompanying

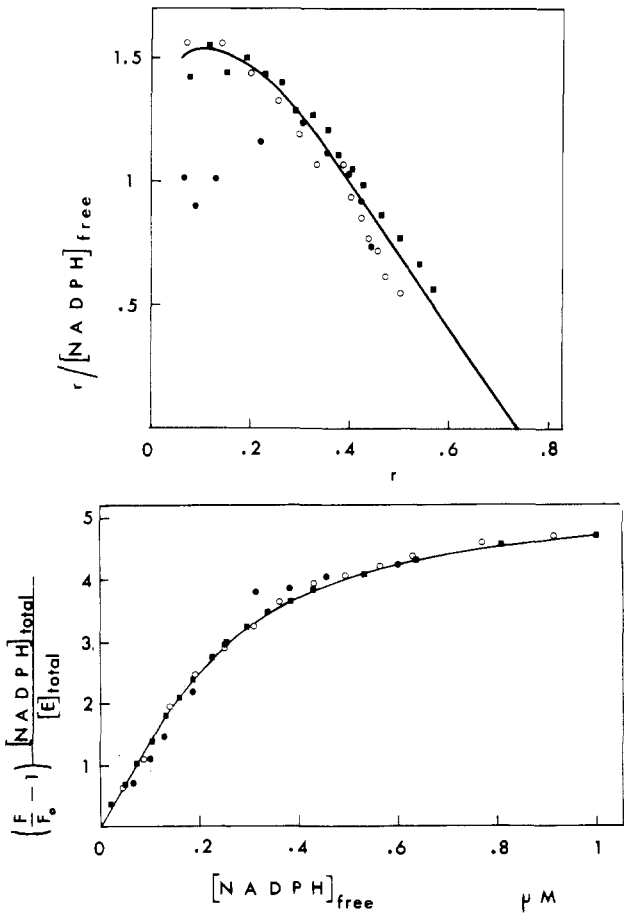

FIGURE 3: Fluorescence titrations of the enzyme by NADPH performed at low enzyme concentration. NADPH (stock solution 0.130 mM) was delivered at the rate of 3.64 μl/min in the cell containing 2.1 ml of buffer P and the enzyme at the following concentrations: (●) 0.17 μM; (○) 0.43 μM; (■) 1.29 μM. (a) Scatchard plots of the data. (b) Fitting of the data to the theoretical curve calculated according to eq 2 with the following parameters: K = 0.5 μM; K' = 0.12 μM, α = 7.7, α' = 6.3.

NADPH binding to the enzyme are quite identical for both diner binding sites.

In order to obtain a more reliable estimation of the dissociation constant, fluorescence titrations were performed in a range of low enzyme concentrations from 0.17 to 1.3 μM, where the monomer species represented 32–15% of total protomer concentration. Under these conditions, free NADPH concentration could be easily measured from the net deviation of the fluorescence titration curve from the stoichiometric straight line obtained when a high enzyme concentration (9 μM) was used. If a linear relation is assumed between the fluorescence changes and the number of bound NADPH molecules, the binding isotherms exhibited positive cooperativity (Figure 3a). This cooperativity, within the experimental error, was independent of the enzyme concentration when higher than 0.4 μM, which indicated that interaction between bound NADPH molecules takes place within the dimer species. Some deviation from the normalized curve occurred, however, for the lowest enzyme concentration tested (0.17 μM), suggesting that binding of NADPH to the monomer species induced the association of the enzyme. Fluorescence titrations at lower enzyme concentrations were hampered by the weakness of the observed fluorescence intensity.

Different models have been elaborated and tested to explain this apparent cooperativity. The most general binding scheme is the following "square" model, where NADPH binds to the dimer molecule, D, on two sites, 1 and 2, with different affinities, K_1 and K_2, and fluorescence enhancements α_1 and α_2. Interaction occurs between the binding sites, and an average fluorescence enhancement, α', is measured when 2 NADPH molecules are bound.

$$\begin{array}{ccc} D & \overset{K_1}{\rightleftharpoons} & DX_1 \\ {\scriptstyle K_2}\updownarrow & & \updownarrow{\scriptstyle K'_2} \\ DX_2 & \underset{K'_1}{\rightleftharpoons} & DX_1X_2 \end{array}$$

The fluorescence intensity of the sample, F, and that of NADPH in the absence of enzyme, F_0, are correlated by the equation:

$$\left(\frac{F}{F_0} - 1\right)\frac{[X]_T}{[E]_T} = \frac{(\alpha_1 - 1)\frac{[X]}{K_1} + (\alpha_2 - 1)\frac{[X]}{K_2} + 2(\alpha' - 1)\frac{[X]^2}{K_1'K_2'}}{2\left(1 + \frac{[X]}{K_1} + \frac{[X]}{K_2} + \frac{[X]^2}{K_1K_2'}\right)} \quad (1)$$

where $[X]_T$ and $[X]$ are the total and free NADPH concentrations, and $[E]_T$ the total concentration of binding sites. Not less than six parameters are necessary for the total description of this model. In the range of low NADPH concentrations, the experimental uncertainties were large, small fluorescence intensities being observed. This could not allow us to improve, with reasonable confidence, such a sophisticated model, to the same extent as that elaborated by Koren and Hammes for S-malate dehydrogenase (Koren and Hammes, 1975). A simplication is introduced if the dimer molecule is supposed to be initially symmetrical. The square model reduces then to a linear one:

$$D \overset{X}{\underset{K}{\rightleftharpoons}} DX \overset{X}{\underset{K'}{\rightleftharpoons}} DX_2$$

and eq 1 can be written:

$$\left(\frac{F}{F_0} - 1\right)\frac{X_T}{E_T} = \frac{(\alpha_1 - 1)\frac{(X)}{K} + (\alpha' - 1)\frac{(X)^2}{KK'}}{1 + \frac{2(X)}{K} + \frac{(X)^2}{KK'}} \quad (2)$$

This simplification was found to be acceptable and four parameters are sufficient to explain our results. Figure 3b shows the fitting of our data to eq 2, with the following values of the parameters: K = 0.5 μM, K' = 0.12 μM, α = 7.7, and α' = 6.3. As a control, it was checked that neither the assumption of $K = K'$ (with $\alpha \neq \alpha'$) nor $\alpha = \alpha'$ (with $K \neq K'$) could fit our data.

The finding that α and α' values differ by only 20% gives an "a posteriori" justification to the assumption of constant fluorescence enhancement in the previous estimation of $(NADPH)_{free}$ from fluorescence titrations. It also explains the agreement observed between ultrafiltration and fluorescence data.

Ternary Complexes Formed by Isocitrate Dehydrogenase with NADPH and Various Ligands (Figure 1). The fluorescence and thermodynamic parameters of the enzyme–NADPH complex were unaffected by the presence of EDTA up to 10 mM, or by the presence of magnesium ions up to 20 mM. Dinucleotides are known to chelate Mg^{2+} ions with reported dissociation constants of 10 mM (Duggleby and Dennis,

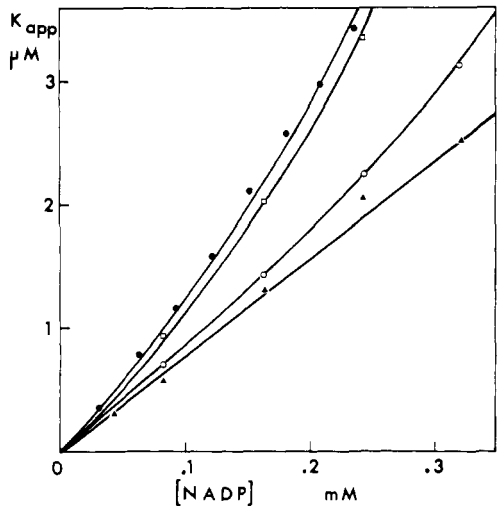

FIGURE 4: Equilibrium fluorescence measurements of the competition between NADPH and NADP. NADP was added to a solution containing enzyme (3.85 μM effective NADPH sites) in phosphate buffer and NADPH at the following concentrations: (●) 8.72 μM; (□) 17.3 μM; (○) 5.85 μM; (▲) 2.92 μM. For each experiment, the value of K_{app} is calculated and plotted against NADP concentration.

TABLE I: Complexes Formed by NADPH with Isocitrate Dehydrogenase in the Presence of Various Ligands.

Complex	Dissociation Constant of NADPH (μM)	Fluorescence Enhancement
E-NADPH	0.5	7.7
	0.12	6.3
E-isocitrate-NADPH	5.5 ± 1	~6.5
E-isocitrate-EDTA-NADPH	>1	~13
E-isocitrate-Mg-NADPH	∞	~2

1970) to 20 mM (Apps, 1973). This binding is weak compared with that of Mn^{2+} in the same complexes (Colman, 1972). Under our experimental conditions, the greatest part of Mg^{2+} ions was chelated by phosphate buffer (K_{ass} = 70 M^{-1}, Bock, 1960), and thus in the 0–20 μM NADPH concentration range, no more than 10% NADPH was calculated to be bound to Mg^{2+}.

In the presence of isocitrate, a decrease was observed in the affinity of NADPH towards the enzyme. The fluorescence level reached at infinite NADPH concentration was that of the enzyme-NADPH complex. The evidence for the formation of a ternary complex E-isocitrate-NADPH was provided by the fact that, when isocitrate concentration was increased, a characteristic NADPH titration curve was obtained, which yielded a value of 5.5 μM ± 1 μM for the apparent dissociation constant of NADPH in this complex.

When the enzyme was titrated by NADPH in the presence of Mg^{2+} and isocitrate, no modification was observed in the fluorescence of NADPH with respect to free coenzyme. This result is in good agreement with the Sephadex G-25 molecular sieving data and shows that a ternary complex does not exist. This point was also verified by ultrafiltration.

In the presence of isocitrate and EDTA, NADPH fluorescence was enhanced about 12–13 times when bound to the enzyme. This enhancement could be accounted for by the tight binding of NADPH in a quaternary complex enzyme-

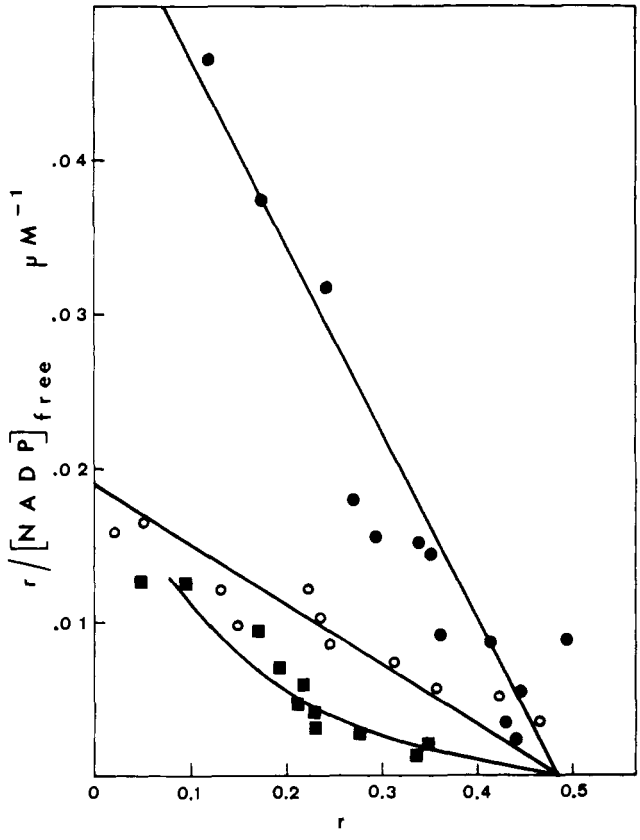

FIGURE 5: Scatchard representation of the binding of NADP to isocitrate dehydrogenase studied by equilibrium dialysis. Experiments were conducted at 4 °C in buffer P at an enzyme concentration of 61 μM with the following additions in the enzyme compartment: (○) none; (●) 30 μM NADPH; (■) 73 μM NADPH.

NADPH-isocitrate-EDTA (K_D > 1 μM). In the presence of 37 mM isocitrate, and at a saturating level of NADPH, EDTA binding in the quaternary complex followed a hyperbolic lay yielding the value of 0.280 mM for the dissociation constant.

In Table I are shown the characteristics of NADPH binding in these complexes.

In the presence of NADP, NADPH bound to the enzyme with apparently decreased affinity. Fluorescence data showed competition between NADP and NADPH. In a competition experiment, NADP was added to the E-NADPH complex at different NADPH saturation levels. Data were plotted according to the following equation which describes a simple competition scheme:

$$K_{app} = \left(\frac{E_{total}}{E\text{–}NADPH} - 1\right) NADPH_{free}$$

$$= K_{NADPH}\left(1 + \frac{NADP_{free}}{K_{NADP}}\right)$$

$$K_{NADPH} = \frac{(E)\cdot(NADPH)}{(E\text{–}NADPH)}$$

$$K_{NADP} = \frac{(E)\cdot(NADP)}{(E\text{–}NADP)}$$

The plot of K_{app} vs. NADP deviated considerably from the theoretical straight line expected (Figure 4). This discrepancy shows that a simple competition model could not account for our results.

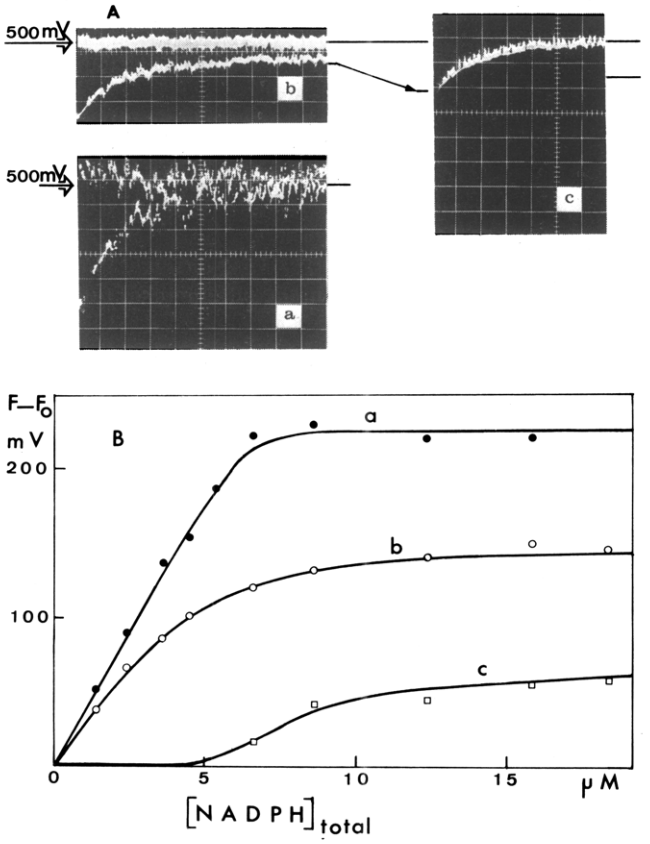

FIGURE 6: Kinetics of NADPH fluorescence enhancement upon binding to isocitrate dehydrogenase, followed in a stopped-flow apparatus. The reaction was performed at 20 °C in buffer P. Enzyme (15.5 μM) was present in syringe 1, either alone (a) or in the presence of DL-isocitrate (10 mM) (b) first step; (c) second step. NADPH concentration was varied in syringe 2. (A) Typical oscilloscope tracings of the reaction of the enzyme with 15.9 μM NADPH. (a) ordinate: 20 mV/division; abscissa 2 ms/division; (b) ordinate 50 mV/division, abscissa 5 ms/division; (c) ordinate 20 mV/division, abscissa 0.1 s/division. Time constant: 0.1 ms. Free NADPH fluorescence level (i.e., level at time zero): 290 mV. Final level 500 mV. (B) Plots of the final fluorescence amplitudes of the association reaction of NADPH with the enzyme vs. final total NADPH concentration.

NADP Binding to Isocitrate Dehydrogenase. NADP binding was studied by equilibrium dialysis using an enzyme concentration of 61 μM. The Scatchard representation (Figure 5) shows that only 0.5 site/protomer was titrated, i.e., 1 binding site/dimer, with a dissociation constant of 26 ± 2 μM. In an analogous experiment, 30 μM NADPH was added to the enzyme compartment, so that 99% NADPH was bound to the enzyme, at the average proportion of 1 molecule of NADPH/dimer. The same number of NADP binding sites was found under these conditions, but a dissociation constant of 7.7 μM was obtained. When the proportion of added NADPH was higher than 1 molecule/dimer, the Scatchard plot exhibited positive concavity, indicating a competition between NADP and NADPH with variation in free NADPH concentration along the binding curve (Best-Belpomme and Dessen, 1973).

Kinetics of the Coenzyme Binding to Isocitrate Dehydrogenase. NADPH association with the enzyme was studied in a stopped-flow apparatus. The increase in fluorescence with time upon binding of NADPH was observed. Two molecules of NADPH apparently reacted with one enzyme molecule through one fast second-order reaction (Figure 6). The time course of this reaction was analyzed on the computer, by a multilinear regression method, according to a reversible second-order process:

$$E + NADPH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{–}NADPH$$

The assumed value for the equilibrium dissociation constant in the calculations was varied from 0.1 to 0.5 μM. This, however, did not change the calculated association rate constant noticeably. From Table II, it appears that an average value of 18 μM^{-1} s^{-1} was obtained for k_1 when NADPH concentration was less than one-half of the protomer concentration, and 26 μM^{-1} s^{-1} at higher NADPH concentrations.

TABLE II: Association Rate Constant of NADPH with Isocitrate Dehydrogenase in the Absence and in the Presence of Isocitrate. Enzyme Concentration was 6.2 μM.

NADPH	k_1 (μM^{-1} s^{-1})	
Concn (μM)	0 Isocitrate	+10 mM Isocitrate
1.4	17.4 ± 2.1	
2.31	16.6 ± 0.5	10.9 ± 0.5
3.65	25.9 ± 3.6	9.2 ± 0.9
4.5	19.5 ± 0.4	10.9 ± 0.4
5.35	27.1 ± 4.1	
6.64	27.6 ± 3.5	10.0 ± 0.0
8.62	24.0 ± 2.0	11.4 ± 0.5
12.35	24.8 ± 1.0	10.0 ± 0.4
18.35		11.0 ± 0.7

When 10 mM DL-isocitrate was present with the enzyme, the binding of NADPH occurred through two consecutive reactions of very different rates. The first step was a fast second-order process. An average value of 10 μM^{-1} s^{-1} was obtained for the association rate constant (Table II). Using the K_{NADPH} value of 5.5 μM previously determined from equilibrium measurements, this gives about 50 s^{-1} for the dissociation rate constant of NADPH from the ternary complex enzyme–isocitrate–NADPH. The associated fluorescence change represented about two-thirds of the total amplitude of the reaction (Figure 6B). The second step was 50 times slower ($t_{1/2}$ = 0.1 s) and its rate appeared independent of NADPH concentration. Its amplitude decreased with enzyme aging. When NADPH concentration was increased up to 20 μM, the total fluorescence change was identical with that observed in the absence of isocitrate.

Binding Kinetics of NADPH to the Enzyme in the Presence of NADP. Enzyme and NADP were preincubated in syringe 1, and NADPH was present in syringe 2. NADPH and enzyme were present in constant and almost equimolar concentrations, whereas NADP concentration was varied. As NADP concentration increased, the rate of reaction of NADPH with the enzyme was slower and the fluorescence level at the end of the reaction was lower. The reaction was not first order. Assuming that NADPH reacts with free enzyme, the variation of the initial slope $(dF/dt)_{t=0}$ with NADP concentration indicates the saturation level of the enzyme by NADP at time zero in the absence of NADPH. This experiment (Figure 7) yielded a dissociation constant of 26 μM for NADP. This value is in good agreement with equilibrium dialysis data. The variation of the fluorescence amplitude at the end of the reaction with NADP concentration indicates the saturation level of the enzyme by NADPH in the presence of NADP. Again, the apparent equilibrium dissociation constant for NADPH was not linearly related to NADP concentration, as should be expected for a competition scheme, but exhibited a parabolic behavior.

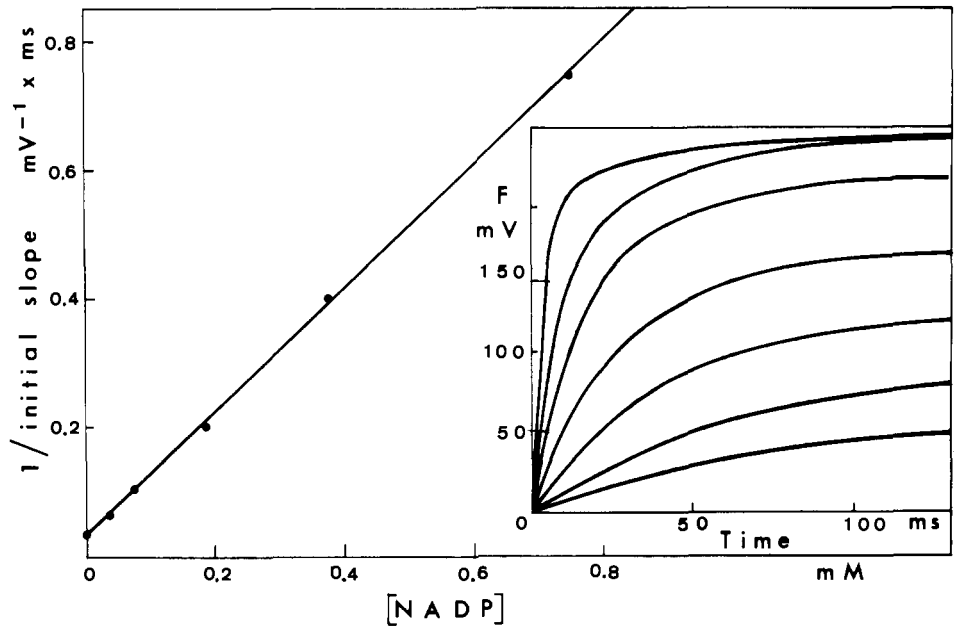

FIGURE 7: Binding of NADPH to the enzyme in the presence of NADP. The experiment was performed under the following conditions: syringe 1: 19,7 μM NADPH. Syringe 2: 16,6 μM enzyme and NADP as follows: downwards: 0; 74,5 μM; 149 μM; 373 μM; 745 μM; 1,49 mM; 2,6 mM. The initial slope was graphically measured, when NADP was present, and calculated using the value of 20 $\mu M^{-1}s^{-1}$ for the second order kinetic constant, in the absence of NADP. This plot yielded the value of 26 μM for the dissociation constant of NADP.

The same kinetics were observed when NADP and NADPH were premixed in syringe 2 and allowed to bind simultaneously to isocitrate dehydrogenase present in syringe 1. This result demonstrates that (1) when NADP concentration was higher than 70 μM, completion of its binding reaction to the enzyme occurred within the dead time of the stopped-flow apparatus, i.e., the association rate constant for NADP is at least 5 μM^{-1} s^{-1}, and (2) the dissociation of NADP from the enzyme was not the limiting step in the formation of the enzyme–NADPH complex, which would lead to first-order kinetics. Indeed, if the equilibrium dissociation constant for NADP is 25 μM, and assuming the value of 5 μM^{-1} s^{-1} for its association rate constant, this yields a minimal value of 125 s^{-1} for the dissociation rate constant.

Dissociation of Bound NADPH by NADP Binding. In this experiment, enzyme–NADPH complex was preformed in syringe 1, in high enough concentrations (20 μM) with respect to the dissociation constants, so that the dissociation of the complex due to dilution proved to be negligible. The NADP concentration in syringe 2 was varied. The dissociation of NADPH resulted in a fluorescence decrease. The observed intensity was corrected for the absorbance of NADP at 340 nm (ΔA = 0.050 mM^{-1} cm^{-1}), which, in the high concentration range used, caused a drop of the intensity at time zero of the reaction (Figure 8). NADPH dissociation consisted of one approximately first-order process. The associated apparent kinetic constant k_0 derived from semilogarithmic plots varied as a decreasing function of NADP concentration and reached the limit value of 2.5 s^{-1} at infinite NADP concentration. This result is in qualitative agreement with a competition model, where the extrapolated value of 2.5 s^{-1} would be the dissociation rate constant k_{-1} of NADPH from the enzyme. Analysis of the final amplitude of the fluorescence decrease yielded an apparently cooperative binding isotherm for NADP. The extrapolated maximal amplitude was smaller than the amplitude expected if NADPH was entirely dissociated from the enzyme.

The simulation of the following competition scheme was undertaken on the computer:

$$E + NADPH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}NADPH \quad (1)$$

$$E + NADP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{\cdot}NADP \quad (2)$$

The experimental initial concentrations of all reagents were used in the calculations, and the following numerical values for the rate constants were assumed: k_1 = 20 μM^{-1} s^{-1}, k_{-1} = 2.5 s^{-1}, k_2 = 5 μM^{-1} s^{-1}, k_{-2} = 125 s^{-1}. In this simulated model, the decrease of the enzyme–NADPH concentration with time does follow apparent first-order kinetics, and the calculated rate constant is a decreasing function of NADP concentration. However, the analysis of the final amplitudes as a function of NADP yields hyperbolic binding isotherms for NADP, NADPH is entirely displaced from its sites by an excess of NADP, and, thus, it appears that this simple competition scheme cannot account for our results. Figure 8 shows how the data deviate from the theoretical curves.

A study of the fluorescence polarization of NADPH when bound to the enzyme was undertaken. The high value of 0.44 found for the polarization of the fluorescence of NADPH in the enzyme–NADPH complex shows that the reduced coenzyme is tightly immobilized on its binding site. This value was independent of the saturation level of the enzyme by NADPH. We could not demonstrate a different value of the polarization when the enzyme was titrated by NADPH in the presence of various NADP concentrations. The same titration curve was obtained whether fluorescence polarization or intensity was observed.

We also attempted to observe possible modifications of the circular dichroism of NADPH in the 340-nm absorption band, upon binding to the enzyme in the presence and absence of NADP. Such a study has been carried out successfully on glutamate dehydrogenase from *Saccharomyces cerevisiae* (Venard et al., 1975). Unfortunately, the obtained signal was

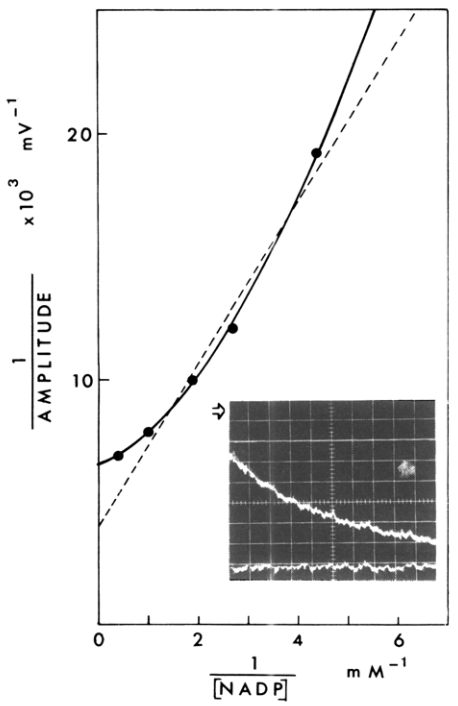

FIGURE 8: Kinetics of binding of NADP to the enzyme–NADPH complex. Enzyme (16.6 μM) and NADPH (20.5 μM) were premixed in syringe 1, and NADP concentration was varied in syringe 2. Reciprocal plots of the final amplitude vs. $NADP^{-1}$. Dashed line: theoretical curve obtained according to the competition model described in the text. Inset: typical recording of the reaction. NADP was 1.25 mM (final concentration). Abscissa: 20 ms/division; ordinate: 20 mV/division. The arrow indicates the fluorescence level when buffer P was substituted to NADP in syringe 2.

too low to work with, even when an enzyme concentration as high as 73 μM was used.

Dissociation of the Enzyme–NADPH Complex by Magnesium–Isocitrate Chelate. The equilibrium results described above predict that dissociation of NADPH must occur when magnesium isocitrate (syringe 1) is mixed with E–NADPH complex (syringe 2). The process of NADPH departure in this case could be compared with the reaction observed where NADP drives NADPH away from the enzyme.

When a solution of Mg^{2+} and isocitrate was rapidly mixed with the preformed E–NADPH(Mg) complex, the observed decrease in fluorescence was found to follow apparent first-order kinetics. The associated kinetic constant, k, varied linearly with magnesium isocitrate concentration. Magnesium ion concentration was buffered in the phosphate medium. The concentrations of free and complexed components of the solutions were calculated using $K_{\text{magnesium isocitrate}} = 0.5\ \text{mM}^{-1}$ (Grzybowski et al., 1970) and $K_{MgHPO_4} = 0.07\ \text{mM}^{-1}$ (Bock, 1960).

A simple interpretation of the linear relationship between k and magnesium isocitrate is the following: the binding of the substrate in a ternary complex occurs through a one-step reversible reaction causing a quenching of NADPH fluorescence. NADPH dissociation takes place after the formation of the ternary complex, according to the scheme:

$$\text{E–NADPH(Mg)} + \text{MgI} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E–NADPH–MgI}$$

$$\text{E–NADPH–MgI} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{E–MgI} + \text{NADPH}$$

Further dissociation of NADPH may occur through a slow approximately first-order ($k_{-2} = 0.6\ s^{-1}$) reaction subsequent to the quenching. k_{-1} and k_1 were determined from our data to be 11 s^{-1} and 0.6 $\mu M^{-1}\ s^{-1}$, respectively, which gives $K_I = k_{-1}/k_1 = 18\ \mu M$, a value about 30-fold higher than the K_m value for this substrate (Carlier and Pantaloni, 1976b).

When 4 mM isocitrate and 10 mM EDTA were rapidly mixed with the enzyme–NADPH complex, the rapid formation (within 5 ms) of a further enhanced fluorescence complex was observed, which is in good correlation with the data from equilibrium experiments.

Discussion

Various equilibrium experiments presented here demonstrate that mammalian cytoplasmic isocitrate dehydrogenase binds 2 molecules of NADPH/dimer enzyme with high affinity and a slight positive cooperativity. Two NADPH binding sites of high affinity have also been found for the cytoplasmic enzyme from pig liver (Illingworth and Tipton, 1970). On the other hand, negative cooperativity occurs in NADP binding to the enzyme. A quite similar behavior is shown by *S*-malate dehydrogenase, which is a dimeric protein exhibiting half-of-the-site reactivity in NADP binding (Tsernoglou et al., 1971), and having nearly equivalent NADPH binding sites (Koren and Hammes, 1975).

It is interesting to compare our results with those obtained by Ehrlich and Colman (1975) on the pig heart enzyme in order to relate possible differences in the mechanism of action of these isoenzymes to their subcellular location and quaternary structure. Indeed, these two isoenzymes of NADP linked isocitrate dehydrogenase have different intracellular distributions in various mammalian tissues, the monomeric mitochondrial species being the only one represented in pig heart (Uhr et al., 1974), whereas the dimeric cytoplasmic protein accounts for 85% of the isocitrate dehydrogenase activity in the liver (Plaut, 1963; Lowenstein and Smith, 1962; Bell and Baron, 1964; Henderson, 1965). These two proteins differ in their physical properties (molecular weight, isoelectric point, extinction coefficient, etc.) and are under independent genetic control (Henderson, 1972). One striking point is that the cytoplasmic enzyme exhibits an affinity towards the coenzyme one order of magnitude higher than the mitochondrial enzyme. This binding result agrees with the data from steady-state kinetics, which show that K_m values are generally lower for the cytoplasmic enzyme (Cleland, 1967; Londesborough and Dalziel, 1970).

The features of NADPH fluorescence change upon binding to the enzyme are similar to those observed for the pig heart enzyme and other NADP linked decarboxylating dehydrogenases, namely, malic enzyme and 6-phosphogluconate dehydrogenase (Silverberg and Dalziel, 1974; Hsu and Lardy, 1967). This indicates that an analogy may exist in the structure of the coenzyme binding sites of this class of dehydrogenases, as has been demonstrated for several NAD dependent dehydrogenases (Branden et al., 1973).

Alteration in the fluorescence of NADPH upon binding to the enzyme in the presence of ligands is a useful tool in the study of ternary complexes. Evidence is given for the existence of a stable enzyme–NADPH–isocitrate complex in which NADPH has a 50-fold lower affinity than in the binary complex. On the other hand, the formation of the complex enzyme–magnesium isocitrate–NADPH can be demonstrated only from rapid kinetics experiments, NADPH and magnesium isocitrate being mutually exclusive on the enzyme, as is the case for the pig heart enzyme. The rates of dissociation of NADPH are very different in all these complexes. This point

must be connected with questions arising from previous results (Carlier and Pantaloni, 1976a,b). The enzyme has been found active towards isocitrate in the absence of divalent metal cations and an activating role of the product NADPH has been demonstrated in the enzymatic catalysis of isocitrate oxidative decarboxylation. This last phenomenon vanished in the presence of divalent metal cations. The catalytic activation by NADPH was then explained within the hypothesis that the rate of NADPH dissociation from the ternary complex enzyme-oxalosuccinate-NADPH had to be faster than the catalytic turnover. In the present work, it is shown that the dissociation of NADPH from the enzyme-isocitrate-NADPH complex is 20-fold faster ($50\ s^{-1}$) than from the binary complex ($2.5\ s^{-1}$) and threefold faster than k_{cat} ($18\ s^{-1}$) (Carlier and Pantaloni, 1976b). If the ternary complex enzyme-oxalosuccinate-NADPH has the same behavior as the enzyme-isocitrate-NADPH complex (which is plausible, since oxalosuccinate is a tricarboxylate-like isocitrate), the present results give some validity to the predicted condition for catalytic activation. The absence of catalytic activation in the presence of magnesium ions may be due to the very slow dissociation rate of NADPH from the complex enzyme-magnesium isocitrate-NADPH. This result had also been predicted.

The following main point to discuss is the question of the asymmetry of isocitrate dehydrogenase. Whereas no asymmetry appears in equilibrium and kinetics data concerning NADPH binding, strong evidence is given for negative cooperativity in NADP binding and for an hybrid complex enzyme-NADP-NADPH in which NADP dissociation constant is identical with the intrinsic dissociation constant K_s derived from steady-state experiments. K_s would not be associated to the equilibrium E + NADP $\rightleftharpoons$ E-NADP ($K = 26\ \mu M$) but rather to another one which could be E-NADPH + NADP $\rightleftharpoons$ E-NADPH-NADP ($K = 8\ \mu M$), and this hybrid complex could be involved in the isocitrate dehydrogenase catalysis. The hypothesis of the occurrence of such a complex had been previously suggested (Carlier and Pantaloni, 1976a) to explain the regulatory role of the ratio [NADPH]/[NADP] by a flip-flop mechanism (Lazdunski et al., 1971).

The problem of the interaction between the coenzymes on the enzyme arises also from binding kinetics experiments: NADPH indeed could not be completely removed from the enzyme by NADP and the plots of the amplitude of the fluorescence decrease vs. NADP showed a cooperative behavior, indicating that a simple competition scheme could not account for the totality of our results. However, ambiguity in interpreting the results comes from the fact that the fluorescence enhancement of NADPH in the eventual E-NADP-NADPH complex is not known. This difficulty could be solved by following NADP binding independently of NADPH fluorescence variation. The fluorescent analogue of NADP, 1-N^6-ethenoadenine dinucleotide phosphate (ϵ-NADP), was prepared for this purpose according to the procedure currently used for the preparation of ϵ-NAD (Barrio et al., 1972; Secrist et al., 1972; Luisi et al., 1975). ϵ-NADP presents the same spectrophotometric properties as ϵ-NAD. Unfortunately, its high fluorescence and very low affinity towards isocitrate dehydrogenase precluded its use in these studies.

We cannot, thus, definitely conclude about the interaction between NADP and NADPH on the enzyme and about the possible involvement of this interaction in the catalytic mechanism. A pre-steady-state study of the reaction, using both absorption and fluorescence detection, is expected to give further knowledge about the various enzyme complexes involved in the catalytic cycle.

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References

Apps, D. K. (1973), *Biochim. Biophys. Acta 320,* 379-387.
Barrio, J. R., Secrist, J. A., and Leonard, N. J. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2039-2042.
Bell, J. L., and Baron, D. N. (1964), *Biochem. J. 90,* 8.
Best-Belpomme, M., and Dessen, P. (1973), *Biochimie 55,* 11-16.
Bock, R. M. (1960), *Enzymes, 2nd Ed. 2,* 3.
Branden, C. I., Eklund, H., Nordstrom, T. B., Soderlund, G., Zeppezauer, E., Ohlsson, I., and Akeson, A. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2439-2442.
Bray, G. A. (1960), *Anal. Biochem. 1,* 279.
Carlier, M. F., and Pantaloni, D. (1973), *Eur. J. Biochem. 37,* 341-354.
Carlier, M. F., and Pantaloni, D. (1976a), *Biochemistry 15,* 1761-1766.
Carlier, M. F., and Pantaloni, D. (1976b), *Biochimie 58,* 27-33.
Carlier, M. F., Pantaloni, D., Branlant, G., and Biellmann, J. F. (1976), *FEBS Lett. 62,* 236-240.
Cleland, W. W. (1967), *Annu. Rev. Biochem. 36,* 107.
Colman, R. F. (1972), *Anal. Biochem. 46,* 358.
Duggleby, B. G., and Dennis, D. T. (1970), *J. Biol. Chem. 245,* 3745-3750.
Grzybowski, A. K., Tate, S. S., and Datta, S. P. (1970), *J. Chem. Soc. A* 241-245.
Ehrlich, R. S., and Colman, R. F. (1975), *Biochemistry 14,* 5008-5016.
Henderson, N. S. (1965), *J. Exp. Zool. 158,* 263-274.
Henderson, N. S. (1972), *Annu. N.Y. Acad. Sci.* 429-440.
Hsu, R. Y., and Lardy, H. A. (1967), *J. Biol. Chem. 242,* 527-532.
Hummel, J. P., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 63,* 530.
Illinworth, J. A., and Tipton, K. F. (1970), *Biochem. J. 118,* 253-258.
Klotz, I. M. (1946), *J. Am. Chem. Soc. 68,* 1486.
Koren, R., and Hammes, G. G. (1975), *Biochemistry 14,* 1021-1025.
Kurganov, B. I., Sugrobova, N. P., and Yakovlev, V. A. (1972), *FEBS Lett. 19,* 308-310.
Lazdunski, M., Petitclerc, C., Chappelet, D., and Lazdunski, C. (1971), *Eur. J. Biochem. 20,* 124-139.
Londesborough, J. C., and Dalziel, K. (1970), *Pyridine Nucleotide Dependent Dehydrogenases, Proc. Adv. Study Inst., 1969,* 315-324.
Lowenstein, J. M., and Smith, S. R. (1962), *Biochim. Biophys. Acta 56,* 385-387.
Luisi, P. L., Baici, A., Bonner, F. J., and Aboderin, A. A. (1975), *Biochemistry 14,* 362-368.
Plaut, G. W. E. (1963), *Enzymes, 2nd Ed. 7,* 105-214.
Rippa, M., Signorini, M., and Dallochio, F. (1974), *FEBS Lett. 39,* 24-26.
Rose, Z. B. (1960), *J. Biol. Chem. 235,* 928-933.
Scatchard, G., Coleman, J. S., and Shen, A. L. (1957), *J. Am. Chem. Soc. 79,* 12-20.
Secrist, J. A., Barrio, J. R., Leonard, N. J., and Weber, G. (1972), *Biochemistry 11,* 3499-3505.

Silverberg, M., and Dalziel, K. (1974), *Arch. Biochem. Biophys. 168,* 646–651.
Steinberg, I. Z., and Schachman, H. K. (1966), *Biochemistry 5,* 3728.
Tsernoglou, D., Hill, E., and Banaszak, L. J. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 171–178.
Uhr, M. L., Thompson, V. W., and Cleland, W. W. (1974), *J. Biol. Chem. 249,* 2920–2927.
Venard, R., Jallon, J. M., Fourcade, A., and Iwatsubo, M. (1975), *Eur. J. Biochem. 57,* 371–378.

Cooperative and Noncooperative Binding of Pyridoxal 5′-Phosphate to Tryptophan Synthase from *Escherichia coli*†

Peter Bartholmes,‡,§ Kasper Kirschner,* and Hans-Peter Gschwind

ABSTRACT: An improved purification procedure for the β_2 subunit of tryptophan synthase from *Escherichia coli* has led to an essentially pure and stable preparation with a specific enzymatic activity that is 30% higher than the previously reported maximum value. Sedimentation analysis shows that the apo-β_2 subunit is monodisperse and dimeric down to a concentration of 0.02 mg of protein/ml. The binding of pyridoxal 5′-phosphate (pyridoxal-P) to the apo-β_2 subunit and to the α_2–apo-β_2 complex was studied by equilibrium dialysis and spectroscopic titration. Both the β_2 subunit and the $\alpha_2\beta_2$ complex bind 2 mol of pyridoxal-P with no unspecific binding observable at higher concentrations of pyridoxal-P. The binding of pyridoxal-P to the apo-β_2 subunit is cooperative (Hill coefficient $n_H = 1.7$). The data have been fitted to the Adair equation, yielding the apparent microscopic dissociation constants for the complexes with one and two bound ligand molecules. They differ by a factor of 38, suggesting that the apo- and holo-β_2 subunits have distinct conformations. The binding of pyridoxal-P to the α_2–apo-β_2 complex is noncooperative with a value of the dissociation constant intermediate between the two values of the β_2 subunit. This finding suggests that the α subunit may stabilize a third conformational state of the β_2 subunit.

The dimeric β_2 subunit of tryptophan synthase from *Escherichia coli* (L-serine hydro-lyase (adding indole) (EC 4.2.1.20)) contains bound pyridoxal 5′-phosphate (pyridoxal-P[1]) which is the coenzyme participating in synthesis of L-tryptophan from L-serine and indole:

$$\text{indole} + \text{L-serine} \longrightarrow \text{L-tryptophan} + H_2O$$

This reaction is also catalyzed by the $\alpha_2\beta_2$ multienzyme complex with a 50-fold increase in turnover number over the isolated β_2 subunit. The literature on this enzyme has been reviewed recently by Yanofsky and Crawford (1972). The catalytic mechanism of tryptophan synthesis is qualitatively the same for the β_2 subunit and the complex (Faeder and Hammes, 1970, 1971). The heterologous interaction between α and β_2 subunits is thought to involve mutually induced conformational changes which are responsible for the observed simultaneous increases in catalytic efficiency and affinity for substrates (Faeder and Hammes, 1971; Kirschner et al., 1975a; Weischet and Kirschner, 1976). In this work, we investigate the equilibrium binding of pyridoxal-P to the β_2 subunit and the $\alpha_2\beta_2$ complex of tryptophan synthase as a means of probing the effects of heterologous protein–protein interaction on the active site.

Materials and Methods

Materials. Pyridoxal-P was purchased from Serva (Heidelberg) and purified by chromatography on Amberlite XE-64 (Peterson and Sober, 1954). Mutant strains of *E. coli* K12 *trp*A2/F′*trp*A2 and *trp*B8 were kindly donated by Drs. C. Yanofsky and I. P. Crawford. The cells were grown in 300- or 1500-l. fermenters in a minimal salts medium essentially as described by Creighton and Yanofsky (1970). The bacterial paste was stored frozen at −18 °C.

Buffers. Unless stated otherwise, all experiments were performed with buffer A: 0.1 M sodium pyrophosphate (pH 7.5) containing 10^{-4} M EDTA and 10^{-4} M 1,4-dithioerythritol. Other buffers can interfere with the binding of pyridoxal-P. For instance, phosphate is sometimes a competitive inhibitor for the binding of pyridoxal-P (Martinez-Carrion, 1975; Gianfreda et al., 1974), whereas Tris forms a Schiff base with the ligand (Simon and Kröger, 1974). Reaction of the carbonyl group with dithioerythritol is negligible (Lienhard and Jencks, 1966). Buffer B: 0.1 M imidazole-HCl (pH 7.0) containing 10^{-4} M pyridoxal-P, 2×10^{-4} M dithioerythritol, 2×10^{-3} M EDTA, and 10^{-4} M phenylmethylsulfonyl fluoride. Buffer C: 0.1 M potassium phosphate (pH 7.8) with 1×10^{-3} M dithioerythritol, 5×10^{-3} M EDTA, and 5×10^{-4} M phenylmethylsulfonyl fluoride. pH measurements of buffered ammonium sulfate solutions were performed at 22 °C after tenfold dilution with water.

Assays. Enzymatic activity and protein concentration were determined as described previously (Kirschner et al., 1975b).

† From the Department of Biophysical Chemistry, Biozentrum der Universität Basel, Klingelbergstrasse 70, CH 4056 Basel, Switzerland. *Received April 5, 1976.* This work was supported by Grants 3.8600.72 and 3.4380.74 of the Schweizerische Nationalfonds (to K.K.).

‡ Present address: Universität Regensburg, Institut für Biophysik. & Phys. Biochemie FBB, Universitätsstrasse 31, D-84 Regensburg, West Germany.

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[1] Abbreviations used are: Pyridoxal-P, pyridoxal 5′-phosphate; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.