DPN-linked Isocitrate Dehydrogenase of Bovine Heart: Structural Changes Associated with Activation by ADP*

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Highly purified preparations of DPN-linked isocitrate dehydrogenase of bovine heart have been found in the analytical ultracentrifuge to contain a component with a sedimentation constant of 10.3 S. The sedimentation pattern is markedly altered in the presence of ADP, which had previously been shown to enhance the activity of the enzyme. With low concentrations of ADP the 10 S component is replaced by one sedimenting at 18.6 S; with higher concentrations of ADP, peaks sedimenting as rapidly as 25.7 S have been observed. These findings indicate that the enzyme is aggregated by ADP, and it has been found that such aggregation is fully reversible. The inhibitor DPNH does not aggregate the enzyme, but prevents it from being aggregated in the presence of ADP. The aggregation by ADP occurs without added metal ions. The protein is not aggregated in the presence of high concentrations of isocitrate and Mn^{2+}. ADP stimulates the enzymic reaction with either Mg^{2+} or Mn^{2+} as the metal cofactor. The values of the Michaelis constant for both Mg^{2+} and Mn^{2+} are markedly decreased by ADP. A short lag period of several seconds can be detected in the deactivation by dilution of ADP-activated DPN-linked isocitrate dehydrogenase of heart at 25°. Binding studies utilizing Sephadex G-100 columns suggest that activation by ADP of DPN-linked isocitrate dehydrogenase is probably associated with binding of ADP itself to the enzyme rather than due to the formation of an enzyme-AMP or a phosphorylated enzyme species. A combination of Mn^{2+} and *threo*-D,-isocitrate protects the enzyme against inhibition by *p*-chloromercuriphenylsulfonate, but the addition of either Mn^{2+} or isocitrate alone affords very little or no protection.

The activity of DPN-linked isocitrate dehydrogenase isolated from bovine cardiac mitochondria was found to be stimulated markedly by ADP (Chen and Plaut, 1962; 1963a), while ATP and DPNH are potent inhibitors competitive with DPN^+. Hence, it seemed possible that the enzyme could be the focus of a mechanism for the regulation of mitochondrial oxidation, whereby activity of the enzyme was dependent on the relative concentrations of these nucleotides in mitochondria. The principal effects of ADP were reported (Chen and Plaut, 1963a) to be stabilization of the enzyme at low ionic strength, a shift of the *p*H optimum from *p*H 6.7 to *p*H 7.2 at relatively low isocitrate concentrations, and a marked increase in the apparent affinity of the enzyme for substrate, indicated by a lowering of the Michaelis constant for isocitrate. ADP has therefore been included in the usual reaction mixture for the assay of the enzyme (Chen and Plaut, 1963a), and was used to stabilize and activate the enzyme system in studies on the stereospecific nature of the hydrogen transfer mediated by DPN-linked isocitrate dehydrogenase (Chen and Plaut, 1963b). The observation that the enzyme is activated and stabilized by ADP has been reported to be confirmed by Goebell and Klingenberg (1963), who have noted that these properties are also exhibited by DPN-linked isocitrate dehydrogenase from mitochondria of tissues other than heart.

Extensive purification of the enzyme (Chen and Plaut, 1963a) followed by ultracentrifugal analysis allowed the identification of DPN-specific isocitrate dehydrogenase with a major component (about 85% of the total protein) having a sedimentation constant ($s_{20,w}$) of 10.3 S. The following evidence was then presented that the activity of the enzyme is associated with this component: (a) A sample of purified enzyme (specific activity, 4500 units per mg of protein) when centrifuged at 59,780 rpm in a Spinco Model E ultracentrifuge exhibited one major (about 85%) and one minor (about 15%) component sedimenting at rates of 10.3 S and 4.6 S, respectively (cf. Fig. 1A). After centrifugation, the top third of the cell contents containing the 4.6 S component was removed with a syringe. It was found that the fluid at the bottom of the cell had 83% of the enzymic activity and a specific activity three times higher than the fluid removed. (b) When the enzyme was prepared by a somewhat different procedure (two hydroxylapatite chromatography steps, but not DEAE-cellulose chromatography) the major component was still found to sediment at 10.3 S, but, the minor component now sedimented at 6.2 S (cf. Fig. 1B). (c) On the basis of a sedimentation constant of 10 S it has been estimated that the molecular weight of the protein is around 300,000. The possibility that the isocitrate dehydrogenase activity is associated with the large molecular weight component (10.3 S) was enhanced, therefore, by the finding that the passage of the enzyme through a column of the molecular sieve Sephadex G-200 was essentially unretarded. The availability of enzyme for sedimentation studies suggested that the ADP effect could be investigated from the point of view of possible aggregation phenomena since another enzyme, liver glutamate dehydrogenase, had been found to be both stimulated and aggregated by ADP (Frieden, 1959a). Also, glucose-6-phosphate dehydrogenase from various

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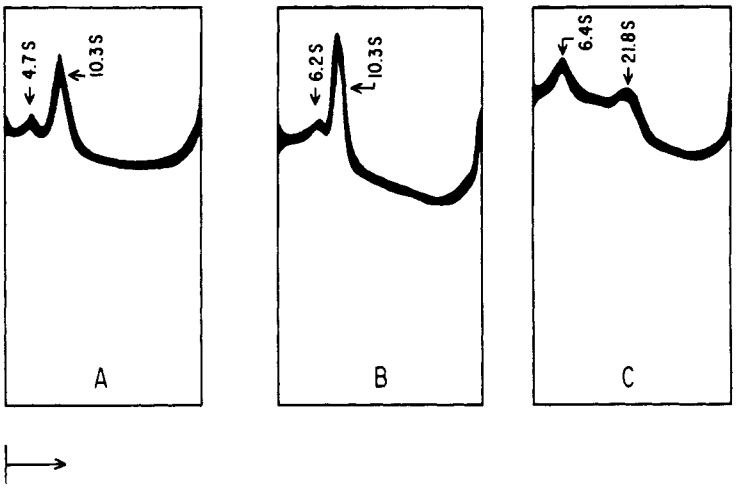

FIG. 1.—Alteration by ADP of sedimentation velocity of DPN-linked isocitrate dehydrogenase. All experiments were done with enzyme which had been dialyzed thoroughly against 0.10 M potassium phosphate buffer, *p*H 7.2. The photographs shown were taken about 16 minutes after reaching a speed of 59,780 rpm and at a phase plate angle of 40–50°. (A) Enzyme prepared by a procedure involving both DEAE-cellulose and hydroxylapatite chromatography (Chen and Plaut, 1963a); specific activity, 4500 units/mg; protein concentration, 4.4 mg/ml. (B) Enzyme prepared by a procedure involving two hydroxylapatite chromatography steps, but not DEAE-cellulose chromatography (Chen and Plaut, 1963a); specific activity, 3200; protein concentration, 7.6 mg/ml. (C) Enzyme preparation: In the analysis of part B, above, 0.8 ml of 1.2 ml of enzyme solution was actually centrifuged. Following the run, enzyme was recovered from the cell and combined with the remaining 0.4 ml of solution. Protein was then precipitated by addition of an equal volume of saturated ammonium sulfate solution, collected by centrifugation, taken up with 0.8 ml of 0.005 M potassium phosphate buffer, *p*H 7.2, and dialyzed against 500 ml of 0.10 M potassium phosphate buffer for 2.5 hours. A slight turbidity was removed by centrifugation. ADP was added to the supernatant to give a final concentration of 6.7×10^{-4} M. Enzyme specific activity, 2500; protein concentration, 8.0 mg/ml.

sources seems to be stabilized and aggregated by the nucleotide TPN^+ (Kirkman and Hendrickson, 1962; Noltmann and Kuby, 1963; Tsutsui and Marks, 1962).

To supplement the previous kinetic studies delineating the activation by ADP, data are now presented indicating that ADP produces marked changes in the sedimentation behavior, suggestive of aggregation of DPN-linked isocitrate dehydrogenase. Evidence has also been obtained which suggests that ADP binds to the enzyme and affects the Michaelis constants for Mn^{2+} and Mg^{2+}.

MATERIALS AND METHODS

DPN-linked Isocitrate Dehydrogenase of Bovine Heart.—The preparation of the enzyme by two slightly different procedures has been described (Chen and Plaut, 1963a). Enzyme preparations with specific activities ranging from 2200 to 4500 units/mg have been used throughout these studies.

Reagents.—[^{32}P]ADP, with the label in the terminal phosphate group, was prepared as previous described (Chiga and Plaut, 1959). [8-^{14}C]ADP, 28.7 mc/mmole, lot 6303, was purchased from Schwarz BioResearch, Inc., Orangeburg, N. Y. Radioactive ADP was conveniently purified by DEAE-cellulose chromatography described by Pastore and Friedkin (1961) for the separation of pyridine nucleotides. This method was found to be highly effective in separating ADP from any contaminating 5′-AMP or ATP. Sephadex G-100 from Pharmacia Fine Chemicals, Rochester, Minn., was equilibrated with buffer and the fines were removed before use. *p*-Chloromercuriphenylsulfonic acid was purchased from Sigma Chemical Co. All other reagents used were as described previously (Chen and Plaut; 1963a,b).

Determinations.—Protein was assayed by the method of Warburg and Christian (1941). Where protein solutions contained DPNH or ADP, protein was determined before addition of the nucleotide and the concentration was calculated by correction for dilution. The spectrophotometric assay of enzyme activity has been described (Chen and Plaut, 1963a), and a unit of enzyme activity is defined as that amount which causes an optical density change of 0.01 at 340 mμ due to reduction of DPN^+ under standard assay conditions using a 3.0-ml reaction mixture in a cuvet of 1.0 cm light path length. Radioactivity was determined by plating samples on planchets and counting in a thin-window gas-flow Geiger counter (Nuclear-Chicago, Model CB-110). Suitable correction for self-absorption was made in the case of ^{14}C determinations.

Ultracentrifugation.—Sedimentation studies were performed in a Spinco Model E ultracentrifuge. Analyses were done at 20° at 59,780 rpm. The sedimentation constants are expressed as $s_{20,w}$; the sedimentation coefficient is at 20°, corrected for a solvent having the density and viscosity of water.

RESULTS

Sedimentation Studies.—Tracings of some sedimentation patterns appear in Figures 1 to 4. Ultracentrifugal analyses of highly purified preparations of DPN-linked isocitrate dehydrogenase consistently revealed a major component sedimenting at 10.3 S. Examples of sedimentation patterns of such preparations are shown in Figure 1A and B, obtained with two enzyme preparations purified by slightly different procedures. Although the minor components of Figure 1A and B have different sedimentation constants, the major components have identical $s_{20,w}$ values. It can be seen (Fig. 1C) that ADP has a very profound effect on the sedimentation pattern obtained with an enzyme preparation essentially the same as that used in the experiment of Figure 1B. The rather broad peak at 21.8 S has

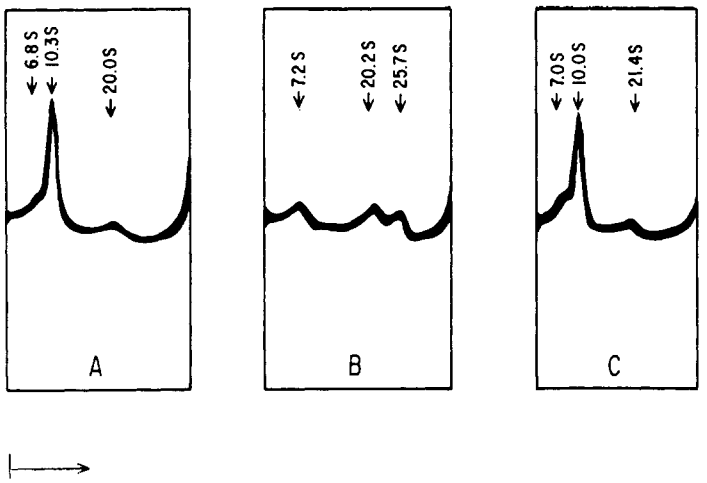

FIG. 2.—Reversibility of ADP-induced aggregation of DPN-linked isocitrate dehydrogenase. The enzyme used in these experiments was prepared in the same manner as the sample shown in Figure 1B. Photographs were taken at about 16 minutes after attaining a speed of 59,780 rpm. Buffer, 0.10 M potassium phosphate, *p*H 7.2. (A) Enzyme preparation (control); no additions; specific activity, 3200; protein concentration, 7.3 mg/ml. (B) Same enzyme preparation as in A, but with 1.0×10^{-3} M ADP; final protein concentration, 6.6 mg/ml. (C) Enzyme solution, which had 1.0×10^{-3} M ADP present as in the preparation of part B above, was precipitated by addition of an equal volume of saturated ammonium sulfate solution, collected by centrifugation, and dialyzed for 2 hours against 500 volumes of 0.10 M potassium phosphate buffer, *p*H 7.2. Specific activity, 2660; protein concentration, 6.1 mg/ml.

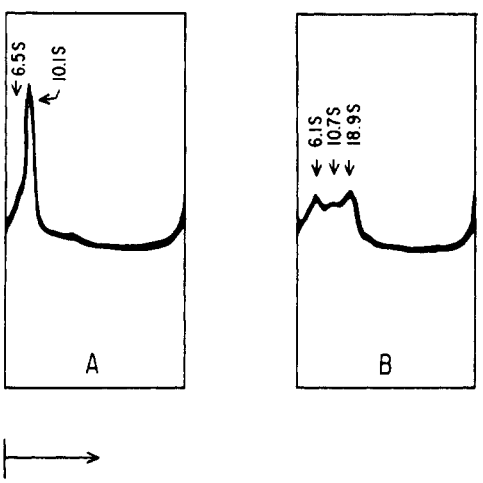

FIG. 3.—Partial aggregation of enzyme by ADP; photographs taken about 8 minutes after reaching speed (59,780 rpm); buffer, 0.10 M potassium phosphate, *p*H 7.2. (A) Control; specific activity, 2300 units/mg; protein concentration, 7.1 mg/ml. (B) Same enzyme preparation but with 1.1×10^{-4} M ADP; specific activity, 2300 units/mg; protein concentration, 6.3 mg/ml.

replaced the 10.3 S component, but the sedimentation rate of the minor contaminating component is scarcely changed. The major component has probably become aggregated.

It was possible that the change could be due to production of a denatured protein in the presence of ADP. However, this appeared unlikely since the enzyme recovered from the cell compartment following ultracentrifugation was highly active. Moreover, if the aggregation were due to denaturation, it might not be reversible; reversibility of the change due to ADP was therefore investigated in the experiments shown in Figure 2. The enzyme preparation used for these experiments contained three easily discernible components (Fig. 2A) comprised of a major peak at 10 S plus small components sedimenting at 7 S and 20 S; the latter were assumed to be impurities. In the presence of 1.0×10^{-3} M ADP (Fig. 2B), the sedimentation pattern was markedly altered by the disappearance of the 10 S peak and the appearance of a broad, rapidly sedimenting component at 25.7 S; the minor components (7 S and 20 S), on the contrary, do not appear to be affected by ADP. Separation of protein from ADP by ammonium sulfate treatment followed by dialysis then resulted in a preparation giving the sedimentation pattern of Figure 2C, which is grossly the same as that obtained before ADP was added insofar, that the major component at 10 S has been restored (see Fig. 2A) while the minor components remain at 7 S and at 20–21 S in all cases (Fig. 2A–C). The aggregation due to ADP was thus readily reversible.

In attempting to correlate the results from such ultracentrifugation studies with kinetic data on the activation by ADP, one should note that the concentrations of ADP used in the experiments of Figure 1 and 2 were in the range where optimal stimulation of initial reaction rates had been obtained (Chen and Plaut, 1963a). These amounts of ADP abolished the component sedimenting at 10 S. If a smaller amount of ADP was used, both the 10 S component and a more rapidly sedimenting species could be detected. In the experiment of Figure 3B, 1.2×10^{-4} M ADP was present; the amount of the original 10 S component (Fig. 3A) has been diminished, and a new species appears which sediments at 18.4 S. The amount of ADP employed apparently determines how much of the 10 S component is altered, and may influence the structure of the new species. Thus, it was found in a number of experiments that the new protein component formed in the presence of 1×10^{-4} M ADP had a sedimentation constant of about 18 S (Fig. 3B), but a more rapidly sedimenting component (26 S) was found when the concentration of ADP was increased to 1×10^{-3} M (Fig. 2B).

DPNH was previously found to be an effective inhibitor of DPN-linked isocitrate dehydrogenase (Chen and Plaut, 1962, 1963a), and it was of interest to determine the effect of this nucleotide on the sedimen-

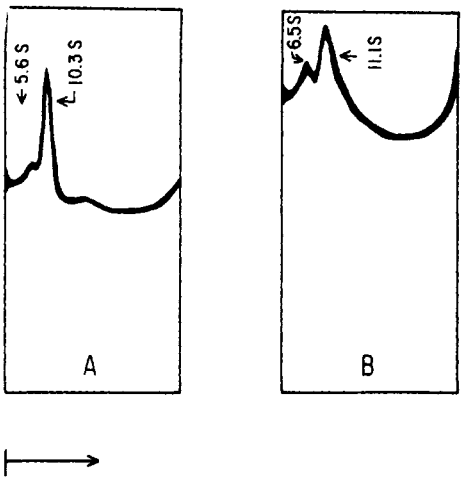

FIG. 4.—The effect of DPNH on the aggregation of DPN-linked isocitrate dehydrogenase; photographs taken about 8 minutes after reaching speed (59,780 rpm) and at a phase plate angle of 45–50°; buffer, 0.10 M potassium phosphate, *p*H 7.2. (A) Enzyme specific activity, 2200 units/mg; protein concentration, 9 mg/ml. DPNH was added to give a final concentration of 3.2×10^{-4} M. (B) The enzyme solution was the same as in part A except that both 3.2×10^{-4} M DPNH and 6.7×10^{-4} M ADP were present.

of the protein. The 10 S component, however, retation behavior of the enzyme. In the experiment of Figure 4A it can be seen that the 10 S component persists in the presence of 3.2×10^{-4} M DPNH, and the pattern obtained was essentially the same as that found in the absence of nucleotide. However, DHNP apparently prevents the aggregation usually produced by 6.7×10^{-4} M ADP (cf. Fig. 1C), since the major component in the presence of both DPNH and ADP was calculated to have a sedimentation coefficient of 11.0 S (Fig. 4B). The amount of DPNH employed (3.2×10^{-4} M) would cause about 70% inhibition of the rate of isocitrate dehydrogenase activity in the usual assay system; however, the extent of inhibition could be varied by changing the concentration of DPN^+, since DPNH is competitive with DPN^+ (Chen and Plaut, 1962, 1963a). The possibility that the 11 S component of Figure 4B represents a denaturation product resulting from incubation of the enzyme with DPNH was ruled out by separate experiments, indicating that loss of enzymic activity was not enhanced by 3.2×10^{-4} M DPNH under the conditions of temperature and ionic strength of the centrifugal studies.

It seems that ADP and DPNH have opposite effects on the sedimentation behavior of the enzyme, just as they do on the kinetic properties. In this connection it should be recalled that DPNH inhibition of enzyme activity was not relieved competitively by ADP (Chen and Plaut, 1963a). This finding would be consistent with the observation illustrated in Figure 4B, assuming that the major component with a sedimentation constant of 11 S represents an enzyme species to which is bound both ADP and DPNH at separate sites, and that such a protein complex is incapable of transformation to the more rapidly sedimenting form.

It should be noted that the ADP-induced aggregation (Figs. 1–3) was produced without added metal ions. One might suspect, therefore, that this ADP effect was not mediated through Mn^{2+} or Mg^{2+}, although enzyme activity itself is completely dependent on added metal ions.

Since ADP influences the apparent affinity of the enzyme for substrate, it seemed possible that substrate itself might influence the sedimentation behavior

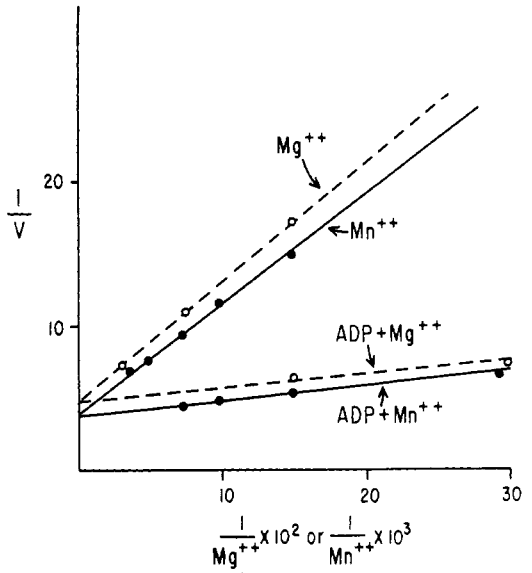

FIG. 5.—Velocity as a function of Mn^{2+} and Mg^{2+} concentration in the presence and absence of 6.7×10^{-4} M ADP. The reciprocal of velocity (V, change in optical density at 340 mμ/minute) is plotted against the reciprocal of metal ion concentration (S, the concentration of metal ion) for experiments done with Mn^{2+} or Mg^{2+}. The conditions for the reactions were those previously described for assay of the enzyme (Chen and Plaut, 1963a,b) except that the metal-ion concentration was varied. In each experiment the cuvet contained 10 μg of protein, specific activity 3000 units/mg.

mained intact in the presence of 2.8×10^{-3} M *threo*-D,L-isocitrate and 1.4×10^{-3} M $MnCl_2$.

Relationship between Metal Ions and the ADP Effect.—It has been established that the activity of DPN-linked isocitrate dehydrogenase of bovine heart depends on the presence of metal ions such as Mn^{2+}, Mg^{2+} (Plaut and Sung, 1954), or Co^{2+}.[1] Since Mn^{2+} is routinely used in the reaction mixture, it was conceivable that stimulation by ADP was mediated through an ADP–metal ion complex. On the other hand, ADP clearly could affect the enzyme without added metal ions, since the changes observed in the ultracentrifuge as well as stabilization at low ionic strength were not dependent on the addition of Mn^{2+} or Mg^{2+}. It seemed possible to test whether or not the activating effect of ADP was mediated through an ADP–metal ion complex by determining whether the same degree of activation occurred with Mg^{2+} as cofactor as with Mn^{2+}. The degree of binding of ADP to these two metal ions differs considerably (Bock, 1960) and this difference might be reflected in the degree of stimulation of the enzyme.

However, the data indicated that ADP activated the DPN-specific isocitrate dehydrogenase system to about the same extent whether the cofactor was Mg^{2+} or Mn^{2+}. In addition, ADP markedly decreased the K_m for added metal ion. The results of such studies are given in Figure 5, where the reciprocal of the velocity is plotted against the reciprocal of the concentration of either Mn^{2+} (Fig. 5A) or Mg^{2+} (Fig. 5B) in the presence and absence of 6.7×10^{-4} M ADP. The Michaelis constant, K_m, for Mn^{2+}, calculated by the method of Dixon (1953), was decreased about 8-fold by ADP, declining from 2.1×10^{-4} M to 2.7×10^{-5} M. The calculated value of V_{max}, however, was the same with and without ADP. In the case of reactions where Mg^{2+} was present in-

[1] A. J. Giorgio, R. C. Chen, and G. W. E. Plaut, unpublished observations (1963).

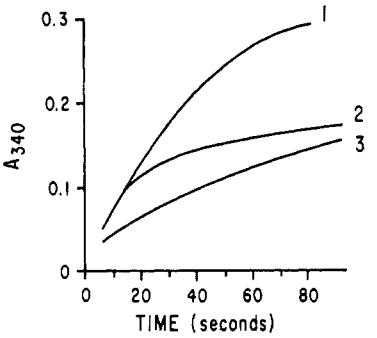

FIG. 6.— Deactivation of DPN-linked isocitrate dehydrogenase. Continuous tracings of reactions followed with a Cary Model 14 recording spectrophotometer at 340 mμ; temperature 25°. All reaction mixtures contained Tris-acetate buffer, *p*H 7.2, 100 μmoles; $MnCl_2$, 0.10 μmole; *threo*-D_sL_s-isocitrate, 16.0 μmoles; DPN^+, 1.0 μmole; 0.1 ml of enzyme solution (10 μg of protein; specific activity, 3000) containing 1×10^{-3} M ADP; and water in a final volume of 3.0 ml. The reaction mixture of experiment 1 had in addition 6.7×10^{-4} M ADP, and the reaction was initiated by addition of the enzyme-ADP solution. In experiment 2 the reaction was also initiated with the enzyme-ADP solution, but no additional ADP was present. In experiment 3 the final contents of the cuvet was exactly as in experiment 2, but the enzyme-ADP solution was mixed into the cell contents 1 minute before initiation of the reaction by addition of the DPN^+. In all cases the last component was added and mixed with the contents of the cuvet with a polyethylene spoon.

stead of Mn^{2+}, the K_m value for Mg^{2+} decreased from 1.8×10^{-3} M to 1.8×10^{-4} M. Again, the apparent V_{max} was the same whether ADP was present or absent. It is interesting to note that while the Michaelis constants for Mg^{2+} were much higher than those for Mn^{2+}, the V_{max} for Mg^{2+} was only 20% lower than that for Mn^{2+}. In the experiments of Figure 5, the concentration of *threo*-D_s-isocitrate employed was 1.67×10^{-3} M, the same relatively high concentration used in the standard assay system (Chen and Plaut, 1963a).

The finding that ADP increases the apparent affinity of enzyme for metal ions is almost identical to observations by Hathaway and Atkinson (1963) in the case of the DPN-linked isocitrate dehydrogenase of yeast: these authors found that 5'-AMP increased the apparent affinity of the enzyme for Mg^{2+}.

These data provide no definitive answer as to whether the activating species is ADP itself or an ADP–metal ion complex. On the other hand, since the association constants of Mn-ADP and Mg-ADP chelates differ markedly (Bock, 1960), and since the activation by ADP is the same regardless of whether Mg^{2+} or Mn^{2+} is the metal cofactor, direct interaction of ADP with the enzyme seems somewhat more probable.

If the action of ADP caused a conformational change in the enzyme molecule resulting in greater availability of the substrate-binding site(s), this might explain the ADP-produced decrease in K_m for both isocitrate and Mn^{2+}. Some evidence has been obtained to indicate that isocitrate and Mn^{2+} do indeed bind at the same site. Thus it has been demonstrated that DPN-linked isocitrate dehydrogenase is sensitive to *p*-chloromercuriphenylsulfonate (Table I), that a combination of isocitrate and Mn^{2+} affords significant protection against inhibition by *p*-chloromercuriphenylsulfonate (experiment 3), and that Mn^{2+} without isocitrate (experiment 2) or isocitrate without added Mn^{2+} (experiment 4) gives very little or no protection.

TABLE I

PROTECTION OF DPN-LINKED ISOCITRATE DEHYDROGENASE BY ISOCITRATE AND Mn^{2+} AGAINST INHIBITION BY *p*-CHLOROMERCURIPHENYLSULFONATE (pCMPS)[a]

Expt.	Reaction Mixture	Reaction Rate[b]	Inhibition (%)
1	pCMPS absent; enzyme added last	215	0
2	pCMPS present; isocitrate added last	0	100
3	pCMPS present; DPN^+ added last	132	39
4	pCMPS present; Mn^{2+} and DPN^+ added last	30	86

[a] Dr. A. J. Giorgio collaborated in the performance of some of these experiments. The reaction mixture of each experiment contained in 3.0 ml: 100 μmoles of Tris acetate buffer, *p*H 7.2; 4.0 μmoles of $MnCl_2$; 16 μmoles of *threo*-D_sL_s-isocitrate; 1.0 μmole of DPN^+; 4.0 μmoles of ADP; water; and enzyme. Where pCMPS was added (experiments 2–4) the final concentration was 6.7×10^{-5} M. Enzyme and pCMPS were incubated for 10 minutes in the presence of all but the missing component(s) of the reaction mixture. The reactions were then initiated by addition of the components lacking from the preincubation mixture. All reactions were carried out at 25°. Enzyme solution (0.1 ml, specific activity, 3000 units/mg), containing 0.3 saturated ammonium sulfate and about 70 μg protein, was added to each cuvet. [b] Expressed as units per ml of enzyme solution.

In experiments similar to those shown in Table I it has been demonstrated that the substrate *threo*-D_s-isocitrate,[2] and not the *threo*-L_s isomer, is responsible for the protection against inhibition by *p*-chloromercuriphenylsulfonate. These results suggest that a functional group at the active site is shielded from reaction with *p*-chloromercuriphenylsulfonate when a *threo*-D_s-isocitrate-Mn complex is already bound to the enzyme. In connection with the decrease of K_m of both the activating cation (Fig. 5) and of isocitrate (Chen and Plaut, 1963a) by ADP it seems significant that the nucleotide does not protect the enzyme against inhibition by *p*-chloromercuriphenylsulfonate when isocitrate is absent from the preincubation mixture. An increase in sensitivity to the sulfhydryl-group binding agent due to the presence of ADP could not be detected.

Reversal of Activation by ADP.—The activation by ADP has been found to be readily reversible. Thus, it has been observed that enzyme preparations which had been activated by ADP could be deactivated merely by separating the protein from ADP by means of ammonium sulfate precipitation or dialysis. These procedures did not permit measurement of the rate of deactivation of the enzyme resulting from removal of ADP. However, one can study this phenomenon if one dilutes a mixture of enzyme and ADP to a concentration where the nucleotide yields only an insignificant amount of stimulation. If the dilution of the combination of enzyme and ADP is made into an isocitrate dehydrogenase assay medium it is possible to follow the time course of the deactivation spectrophotometrically. The isocitrate dehydrogenase assay medium which was used in such studies here contained levels of DPN^+ and isocitrate which should have permitted the reactions to proceed at nearly maximal rates, but Mn^{2+} was limited at a concentration where

[2] We wish to thank Dr. H. B. Vickery for a sample of *threo*-D_s-isocitrate.

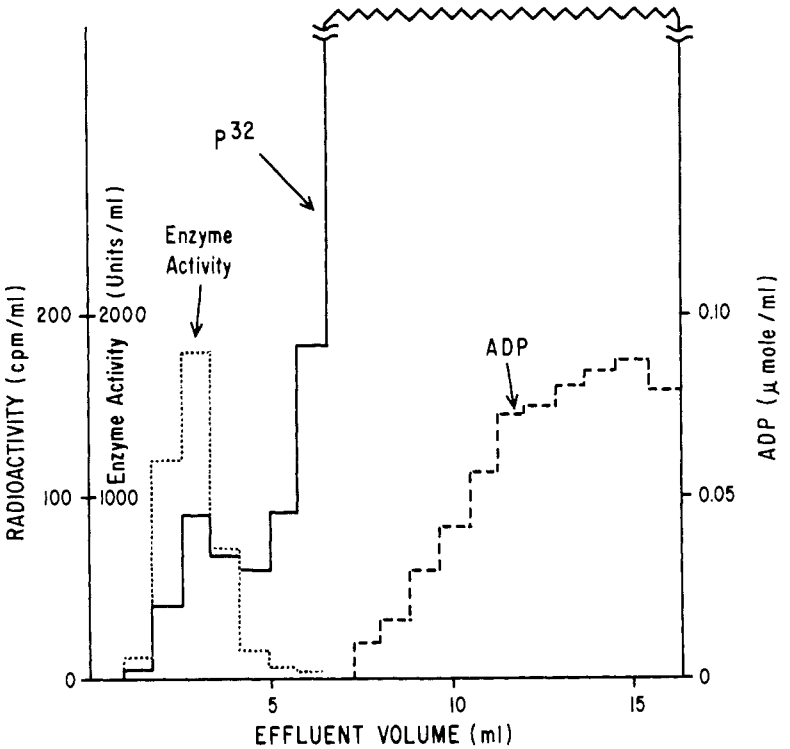

FIG. 7.—Chromatography of a solution containing [^{32}P]ADP and enzyme on Sephadex G-100. A solution of DPN-linked isocitrate dehydrogenase was treated with ammonium sulfate to precipitate the enzyme at 0.5 saturation. The precipitate was taken up in 1.0 ml of 0.0064 M [^{32}P]ADP, specific radioactivity 230,000 cpm/μmole. The resulting enzyme solution had a specific activity of 3000 units/mg and contained 1.0 mg of protein. The solution was placed on a Sephadex G-100 column (1.0 × 17 cm) previously equilibrated with 0.10 M potassium phosphate buffer solution at *p*H 7.2. The column was then developed with the same buffer, and fractions of about 0.8 ml were collected every 6 minutes. The volume of each fraction was determined gravimetrically, assuming a specific gravity of 1.0 g/ml. The radioactivity is represented by the solid line, while enzyme activity is indicated by the dotted line. The broken line represents ADP as determined by the optical density at 260 mμ.

only the *activated* enzyme has sufficient affinity for Mn^{2+} to give a high reaction rate. Typical results of such experiments are shown in Figure 6. In each of the three reactions shown (curves 1–3), 0.1 ml of the enzyme solution which contained 1×10^{-3} M ADP was diluted to a final volume of 3.0 ml. The ADP added with the enzyme was therefore diluted to 3.3×10^{-5} M, a level which yields an insignificant amount of stimulation. In the experiments shown by curves 1 and 2, the reaction was initiated by addition of the enzyme-ADP solution. In the reaction shown by curve 1, 6.7×10^{-4} M ADP was also present in the *final* reaction mixture, but in the experiment represented by curve 2 no ADP was present except for that added with enzyme. It can be seen that the initial slopes of curves 1 and 2 are identical, but the rate of reaction falls off more rapidly with time in the experiment shown by curve 2. In the reaction shown by curve 1, where 6.7×10^{-4} M ADP was present in the final reaction mixture, the decrease in rate with time was due to product inhibition by DPNH (Chen and Plaut, 1963a). However, the more pronounced decline in rate with time observed in curve 2 of Figure 6 must be attributed to deactivation of the enzyme, which was achieved by dilution of the ADP with which the protein was previously in contact, as well as product inhibition by DPNH. A further experiment (Fig. 6, curve 3) showed that an initial rate of reaction slower than that found in curve 2 was obtained when the enzyme-ADP solution was added to the incubation mixture about 1 minute *before* initiation of the reaction with DPN^+. Since little or no difference in initial rates is noted in the isocitrate dehydrogenase assay whether the reaction is started by DPN^+, isocitrate, or enzyme, one may conclude that the enzyme of the experiment shown in curve 3 (Fig. 6) had already been deactivated by the time the reaction was initiated. This conclusion was strengthened when it was found that results identical to those shown in curve 3 could also be obtained by using enzyme which had *not* been preincubated with ADP, but which was added to a reaction mixture containing 3.3×10^{-5} M ADP. Thus, the initial rates of reaction shown in curves 2 and 3 are quite different, although the final reaction mixtures contained the same reagents. While available instrumentation did not allow for convenient study of the detailed kinetics of the deactivation process, one may estimate that deactivation at 25° under the conditions of the experiments shown in Figure 6 is essentially complete within 20 seconds. This is somewhat slower than the rate of activation of the enzyme by ADP which probably occurs within 3 seconds at 25° (Chen and Plaut, 1963a).

The stimulation by ADP has been postulated to be the basis of a metabolic control mechanism in mitochondria (Chen and Plaut, 1963a) and it would seem that the rapidity of activation and deactivation of the enzyme would be consistent with such a concept. If the oxidation of certain substrates were limited by the activity of DPN-linked isocitrate dehydrogenase, the rapid response of the enzyme to fluctuating levels of ADP would allow for a sensitive regulatory system.

Binding of ADP to Enzyme.—The activation of DPN-linked isocitrate dehydrogenase by ADP was

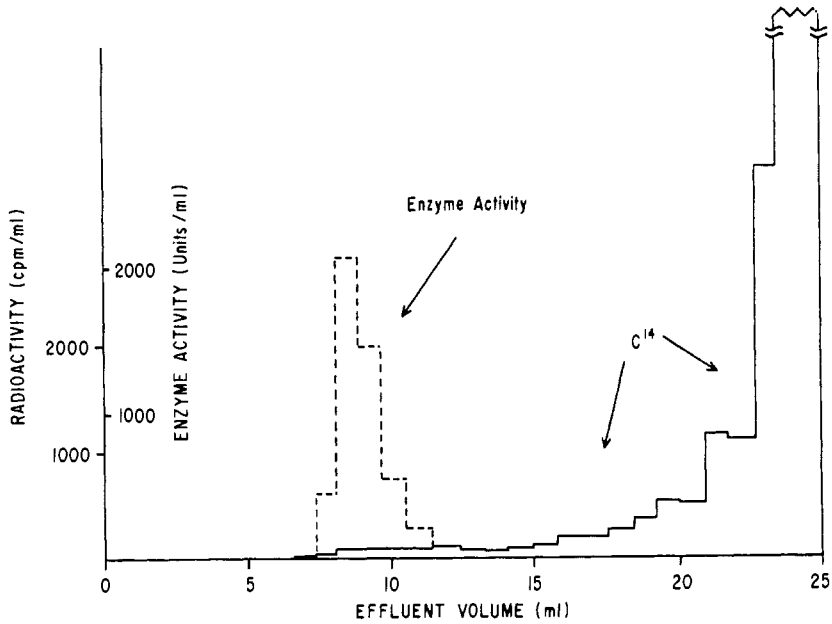

FIG. 8.—Chromatography of a solution containing [8-^{14}C]ADP and enzyme on Sephadex G-100. Enzyme (4000 units; specific activity, 3000) was precipitated by addition of ammonium sulfate, collected by centrifugation, and dissolved in 0.8 ml of 3.5×10^{-4} M [8-^{14}C]ADP (specific radioactivity, 3.09×10^{6} cpm/μmole). The solution was then placed on an 0.8×48-cm Sephadex G-100 column previously equilibrated with 0.10 M potassium phosphate buffer. The column was developed with the same buffer and 0.8-ml fractions were collected every 6 minutes. The dotted line indicates enzymic activity while the solid line represents radioactivity.

studied further to ascertain whether the activated enzyme species was an enzyme-ADP complex or whether ADP actually reacted with the enzyme to produce either an adenyl-enzyme or a phosphorylated enzyme. When a mixture of [^{32}P]ADP and enzyme was passed through a short (1.0×17 cm) column of Sephadex G-100, separation of enzyme from the bulk of ADP clearly occurred, but radioactivity was also bound to the enzymically active proteins (Fig. 7). Calculation of the ratio of ^{32}P to enzyme showed that three fractions with most of the enzyme activity contained 2.3, 3.5, and 6.6 moles of ^{32}P per mole of enzyme, assuming a turnover number of 8×10^{3} moles of DPNH formed per minute per mole of enzyme (Chen and Plaut, 1963a).

Subsequently, a longer Sephadex G-100 column, 0.8×48 cm, was employed. A mixture of enzyme and [^{32}P]ADP (4.4×10^{6} cpm/μmole) was resolved in the same way as described for the experiment of Figure 7. With this larger column it was found that radioactivity was still present in the enzyme-containing fractions, but the molar ratio of ^{32}P to enzyme was only between 1:30 and 1:18. Thus, it appeared that the binding of ^{32}P to protein observed in the experiment of Figure 7 was quite loose.

Since the reactions with [^{32}P]ADP would not have detected the formation of an adenyl-enzyme, a similar experiment was performed with ADP-8-^{14}C. In the experiment shown in Figure 8, the two most active enzyme fractions from the Sephadex column had a ratio of ^{14}C to enzyme of only 1:35 and 1:24. These results again suggest that the degree of binding of ADP to enzyme is weak and the formation of an adenyl-enzyme appears unlikely. Although the formation of a covalent bond during the interaction of ADP and enzyme is not strictly eliminated the possibility seems quite remote, since resolution can be achieved by treatment with Sephadex, a very mild procedure.

DISCUSSION

The data presented suggest that ADP binds to DPN-linked isocitrate dehydrogenase and causes a marked structural change, evidenced by the appearance of rapidly sedimenting components in ultracentrifugal analysis. These data are reminiscent of studies on another enzyme stimulated by ADP, i.e., beef liver glutamate dehydrogenase (Frieden, 1959a). This enzyme was found to have a molecular weight of about 1×10^{6} and a sedimentation coefficient of 26 S (Olson and Anfinsen, 1952). Dissociation of the molecule into subunits having an $s_{20,w}$ between 12 and 13 S occurs on dilution (Olson and Anfinsen, 1952; Kubo *et al.*, 1959), treatment with various inhibitory compounds (Frieden, 1959b; Yielding and Tompkins, 1962), or treatment with 10% dioxane (Churchich and Wold, 1963). The molecular weight of the subunit has been estimated to be about 300,000. Originally it was thought that only the aggregated species was active, and the inhibition by DPNH could be correlated with dissociation into "inactive" subunits. This idea is no longer tenable, since it has been shown that the subunit is active (Fisher *et al.*, 1962; Frieden, 1963; Churchich and Wold, 1963). The molecular weight of glutamate dehydrogenase should more correctly, therefore, be calculated on the basis of a sedimentation coefficient of 12–13 S.

The association-dissociation phenomena in the case of DPN-linked isocitrate dehydrogenase and glutamate dehydrogenase differ in one obvious respect. The former enzyme appears to have little tendency to aggregate except in the presence of ADP, whereas glutamate dehydrogenase associates as a polymer in the absence of ADP as long as the enzyme is present in rather high concentration (Olson and Anfinsen, 1952). Since DPN-linked isocitrate dehydrogenase is active in the absence of ADP, it is highly probable that aggregation is not required for activity. Even

in the presence of ADP the enzyme might well be in the dissociated form at the low protein concentrations used in kinetic studies. Aggregation of the enzyme by ADP may be considered an indication that some structural change is induced by the nucleotide which favors association of several protein units. It is interesting that such structural change is apparently different from that caused by DPNH, which is not an activator but rather an inhibitor. Thus the data presented show that DPNH prevents aggregation by ADP. A similar correlation between prevention of aggregation and inhibition was found for glutamate dehydrogenase inhibition by DPNH by Frieden (1959b). The fact that such correlations may be made may be purely coincidental, but it is noteworthy that such marked effects on large proteins can be produced by relatively small molecules such as DPNH and ADP.

The data presented here for DPN-specific isocitrate dehydrogenase of bovine heart are necessarily more limited than obtainable for commercially available bovine liver glutamate dehydrogenase. Although highly purified DPN-specific isocitrate dehydrogenase can be isolated in greater than 50% yield (Chen and Plaut, 1963a), the amount originally extractable from mitochondrial acetone powder is quite small. Due to scarcity of the enzyme, accurate molecular weight measurements have not yet been reported. On the basis of an $s_{20,w}$ of 10.3 S it was previously estimated that the molecular weight of the enzyme might be 300,000–400,000 (Chen and Plaut, 1963a). Depending on factors such as the shape and degree of hydration, the molecular weight might well be found to be lower. Indeed it should be noted that the sedimentation coefficient ($s_{20,w}$) of 10.3 S for DPN-linked isocitrate dehydrogenase is lower than that of the glutamate dehydrogenase subunit (12–13 S).

It has been claimed that prior treatment and age of the glutamate dehydrogenase preparations influence its physical characteristics (Churchich and Wold, 1963). Such may also be the case for DPN-linked isocitrate dehydrogenase. Although systematic investigation of this point has not been performed, it may be pertinent that stimulation of isocitrate dehydrogenase activity was obtained with enzyme fractions at all stages of purification. Nevertheless, the preparation of the enzyme from heart involves some relatively harsh treatment including (a) exposure to an organic solvent during the preparation of acetone powder and (b) heating at 50° in 0.3 saturated ammonium sulfate solution. The latter treatment actually seems to result in an increased activity yield (Chen and Plaut, 1963a) by a mechanism which is not yet clear. The finding that the enzyme could be heated under conditions of high ionic strength was unexpected since the protein seemed extremely unstable in more dilute solutions,[3] and one may speculate that the treatment might have some effect on the physical behavior of the protein. The data obtained by ultracentrifugal analysis reported here, therefore, should be considered to apply only to protein prepared as described (Chen and Plaut, 1963a).

DPN-specific isocitrate dehydrogenase of bovine heart must be classed with a number of enzymes which are aggregated by activating compounds. Aside from glutamate dehydrogenase, these enzymes include glucose-6-phosphate dehydrogenase of erythrocytes (Kirkman and Hendrickson, 1962; Tsutsui and Marks, 1962) and of yeast (Noltmann and Kuby, 1963), which has been found to be aggregated by TPN^+. Vagelos *et al.* (1963) have also obtained evidence that aggregation may accompany the activation of adipose tissue acetyl-CoA carboxylase by citrate. Thus while aggregation induced by an activator is probably more common than realized, the significance of the aggregation is not clear. If the association of DPN-linked isocitrate dehydrogenase were intimately connected with the observed kinetic effects of ADP, one might postulate that the structural or configurational change in the protein must not only favor aggregation but also make the active site more available to binding with Mn^{2+} (or Mg^{2+}) and isocitrate. The concentrations of ADP needed to produce complete association of the 10 S component roughly parallel the amounts needed for optimal stimulation of the enzyme, so that aggregation may indeed be concerned with the activation by ADP.

The evidence that ADP binds to the enzyme, that such binding may be related to marked configurational changes in the protein, and that ADP alters the affinity of enzyme for activating metal ions and for isocitrate all suggest that the ADP effect is unique rather than nonspecific, and further enhances the possibility that ADP may have a regulatory role regarding the activity of the enzyme in mitochondria.

[3] In preliminary experiments in this laboratory it has been found that the yeast DPN-linked isocitrate dehydrogenase also survives heating at 50° in concentrated solutions of ammonium sulfate, although the enzyme is likewise quite unstable at low ionic strength (Kornberg and Pricer, 1951).

References

Bock, R. M. (1960), *Enzymes 2*, 3.

Chen, R. F., and Plaut, G. W. E. (1962), *Fed. Proc. 21*, 244.

Chen, R. F., and Plaut, G. W. E. (1963a), *Biochemistry 2*, 1023.

Chen, R. F., and Plaut, G. W. E. (1963b), *Biochemistry 2*, 752.

Chiga, M., and Plaut, G. W. E. (1959), *J. Biol. Chem. 234*, 3059.

Churchich, J. E., and Wold, F. (1963), *Biochemistry 2*, 781.

Dixon, M. (1953), *Biochem. J. 55*, 170.

Fisher, H. F., Cross, D. G., and McGregor, L. L. (1962), *Nature 196*, 895.

Frieden, C. (1959a), *J. Biol. Chem. 234*, 815.

Frieden, C. (1959b), *J. Biol. Chem. 234*, 809.

Frieden, C. (1963), *Biochem. Biophys. Res. Commun. 10*, 410.

Goebell, H., and Klingenberg, M. (1963), *Biochem. Biophys. Res. Commun. 13*, 209.

Hathaway, J. A., and Atkinson, D. E. (1963), *J. Biol. Chem. 238*, 2875.

Kirkman, H. N., and Hendrickson, E. M. (1962), *J. Biol Chem. 237*, 2371.

Kornberg, A., and Pricer, W. E., Jr. (1951), *J. Biol. Chem 189*, 123.

Kubo, H., Iwatsubo, M., Watari, H., and Soyama, T (1959), *J. Biochem. (Tokyo) 46*, 1171.

Noltmann, E. A., and Kuby, S. A. (1963), *Enzymes 7*, 223

Olson, J. A., and Anfinsen, C. B. (1952), *J. Biol. Chem. 197* 67.

Pastore, E. J., and Friedkin, M. (1961), *J. Biol. Chem. 236* 2314.

Plaut, G. W. E., and Sung, S.-C. (1954), *J. Biol. Chem. 207* 305.

Tsutsui, E. A., and Marks, P. A. (1962), *Biochem. Biophys Res. Commun. 8*, 338.

Vagelos, P. R., Alberts, A. W., and Martin, D. B. (1963) *J. Biol. Chem. 238*, 533.

Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.

Yielding, K. L., and Tompkins, G. M. (1962), *Recent Progr Hormone Res. 18*, 467.