Biochimica et Biophysica Acta, 429 (1976) 635—644

BBA 67813

STUDIES ON INOSINE MONOPHOSPHATE DEHYDROGENASE. AN ASSOCIATING-DISSOCIATING SYSTEM

ELIZABETH HEYDE and JOHN F. MORRISON

Biochemistry Department, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. (Australia)

(Received October 13th, 1975)

Summary

The techniques of polyacrylamide gel electrophoresis, sedimentation velocity and frontal analysis on Sephadex have been used to demonstrate that preparations of IMP dehydrogenase (IMP: NAD^+ oxidoreductase, EC 1.2.1.14) from *Aerobacter aerogenes* consist of a mixture of molecular weight isomers. Further, it has been shown that dissociation of the higher molecular weight forms is promoted by urea, sodium dodecyl sulphate and dithiothreitol. Under conditions comparable to those used for kinetic analyses, the enzyme has a molecular weight of about 86 000 and this is the smallest active species that has been observed. In the absence of a reducing agent, the enzyme undergoes polymerization and is devoid of catalytic activity. From the amino acid composition and peptide map, it appears that the molecule with a molecular weight of 86 000 is made up of two identical polypeptide chains.

Introduction

IMP dehydrogenase (IMP: NAD^+ oxidoreductase, EC 1.2.1.14), from *Aerobacter aerogenes* has previously been studied by Hampton and co-workers [1], who showed that preparations contained a number of molecular forms with enzymic activity. The aim of the present study was to elucidate the relationships between the molecular forms of the enzyme. As the purified enzyme was to be used for a detailed kinetic study of the reaction mechanism [2,3], it was important to establish that, under the experimental conditions, either a single molecular form of enzyme was present or that different forms exhibited virtually the same kinetic parameters. The requirement of a relatively high concentration of a reducing agent for optimum activity indicated that an investigation of the mechanism of activation by such compounds would also be of interest.

Materials and Methods

IMP and NAD were purchased from P-L Biochemicals Inc., and the concentrations of solutions of these reactants were checked enzymically using IMP dehydrogenase. Tris(hydroxymethyl)aminomethane (Tris) and lyophilized crystalline bovine serum albumin were the products of the Sigman Chemical Company. Dithiothreitol came from Calbiochem; ascorbate and potassium cyanide from British Drug Houses Ltd.; β-mercaptoethanol from Koch-Light Laboratories Ltd.; potassium chloride was a Univar analytical reagent from Ajax Chemicals Ltd., Sydney. Sephadex G200 and Dextran Blue 2000 were purchased from Pharmacia Fine Chemicals.

IMP dehydrogenase was prepared from *A. aerogenes*, Strain P-14, as described by Brox and Hampton [1]. The enzyme was stored at approx. 3 mg protein per ml at −15°C in 0.02 M Tris · HCl buffer, pH 8.1.

Polyacrylamide gel electrophoresis. Electrophoresis was carried out according to Davis [4], using a 7.5% polyacrylamide resolving gel, 0.05 M Tris/glycine buffer (pH 8.9) as the upper buffer and 0.1 M Tris · HCl buffer (pH 8.1) as the lower buffer. Protein was stained with 0.7% amido black in 7% acetic acid, and destained in 7% acetic acid.

Frontal analysis. Frontal analysis was performed using a Pharmacia chromatographic column (0.9 × 30 cm) containing Sephadex G200 which had been swollen for 4 h at 100°C in the appropriate buffer. Fractions of approx. 0.5 ml were collected manually, weighed to determine their volume accurately and then assayed for IMP dehydrogenase activity under the standard conditions given below. The void volume of the column was determined by passing 0.5 ml of Blue Dextran 2000 at low concentration (0.5 mg/ml) through the column, and measuring the absorbance of the effluent fractions at 670 nm. Values for K_{av} were calculated as described by Andrews [5].

Measurement of enzyme activity. Assays were performed by continuously recording the increase in absorbance 340 nm due to the formation of $NADH_2$. For standard assays, each cuvette (1.0 cm light path) contained 0.1 M Tris · HCl buffer (pH 8.1), 1.0 mM NAD, 0.5 mM IMP, 100 mM KCl and 5 mM dithiothreitol in a final volume of 1.0 ml. The mixture was preincubated for 5 min at 37°C in the thermostatted cell holder, fitted with masks, of a Cary Model 14 spectrophotometer, and the reaction was then initiated by the addition of enzyme or IMP. The latter procedure was followed when assaying diluted enzyme or fractions obtained during frontal analysis. The steady-state velocity was calculated from the slope of the linear part of the trace recording change in absorbance at 340 nm.

Protein. Protein was estimated by the method of Lowry et al. [6] using lyophilized crystalline bovine serum albumin as the standard.

Results

Gel electrophoresis of purified IMP dehydrogenase

Following polyacrylamide gel electrophoresis, the purified enzyme was shown to contain 4—5 protein species, with the intensity of the stained protein bands decreasing from the fastest to the slowest moving component. Each of

the bands exhibited enzymic activity. The observations differ from those of Brox and Hampton [1] with respect to the order of the intensity of the stained bands and the absence of faster-moving proteins which are devoid of enzyme activity. When the enzyme was treated with 3 M urea for 30 min and then subjected to electrophoresis, only a single distinct protein band was detected. After removal of the urea by dialysis for 4 h against 0.02 M Tris · HCl buffer (pH 8.1) at 4°C, multiple bands were again apparent on electrophoresis but the relative intensity of the bands was reversed compared with the pattern for the untreated enzyme. The enzyme is inactive in the presence of 3 M urea, but regains 80—100% of its activity after the removal of the urea by dialysis. Essentially one band staining for protein has also been observed on sodium dodecyl sulphate electrophoresis under the conditions of Shapiro et al. [7].

The aforementioned findings suggested that IMP dehydrogenase exists as a mixture of molecular weight isomers. This conclusion was confirmed by the results obtained when the migration of the protein bands was determined as a function of the gel concentration according to the procedure of Hedrick and Smith [8]. Thus, a plot of the mobility of each species against gel concentration gives rise to a family of straight lines that have a common point of intersection at low gel concentration (Fig. 1). The molecular weights of the isomers of IMP dehydrogenase were calculated from a plot of the slopes of the lines in Fig. 1 against molecular weight, using the data given in Fig. 9 of the paper by Hedrick and Smith [8]. The molecular weights obtained were 490 000,

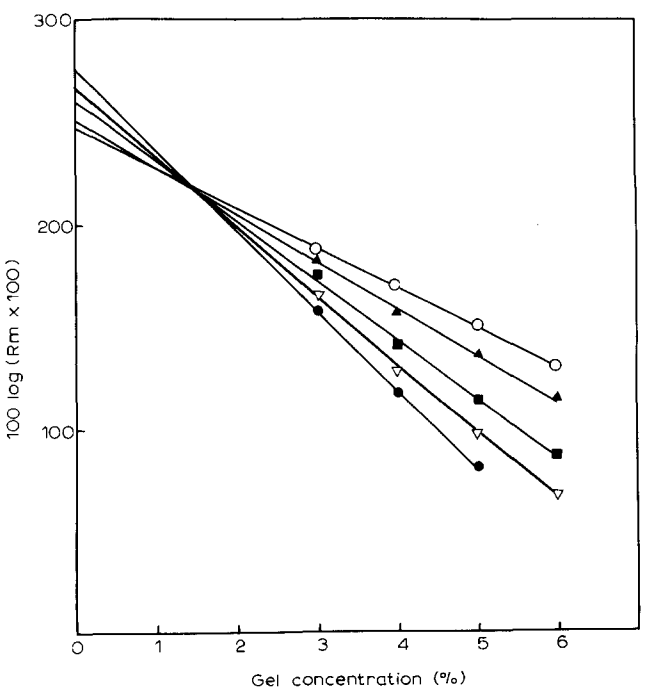

Fig. 1. Effect of different gel concentrations on the mobility of IMP dehydrogenase isomers. Gels were prepared and run as described under Methods. Bromphenol blue was used as a marker, and the mobilities of the enzyme species are expressed relative to the mobility of the marker to give the values for the relative mobility, R_m.

600 000, 780 000, 900 000 and 1 120 000. These can be regarded only as approximate values becuase the range of molecular weights of the isomers extends well beyond that of the standards used. If a deviation of 5% in the estimates is considered, then it seems reasonable to conclude that the various species are multiples of a basic unit with a molecular weight of 160 000, which is approximately twice the molecular weight of the species detected by frontal analysis (see below).

Chromatography of enzyme on Sephadex G200

Attempts to obtain a single component by chromatographing the enzyme preparation at 4°C on a column of Sephadex G200 (2 × 50 cm) in the presence of 0.38 M Tris · HCl buffer (pH 8.1), i.e. the buffer used in the resolving gel for electrophoresis, were unsuccessful. Thus protein appeared in fractions ranging between the void and total volumes of the column. When the last few fractions to emerge from the column were pooled, concentrated and subjected to electrophoresis, the pattern was the same as that obtained with the starting material. The latter result confirms that the IMP dehydrogenase preparation consists of molecular weight isomers which interconvert to re-establish an equilibrium over the experimental period of approximately 24 h. The equilibrium cannot be established rapidly under these conditions because separation of the protein species can be achieved by polyacrylamide gel electrophoresis or in the ultracentrifuge.

Determination of sedimentation coefficients

Three of the components were observed during a sedimentation velocity experiment at 20°C when a solution of the enzyme (3 mg/ml) was centrifuged in the presence of 0.38 M Tris · HCl buffer (pH 8.1). The sedimentation coefficients of the three species were 7.4 S, 10.7 S and approx. 13.8 S. These values are similar to those predicted for a system containing monomer, dimer and trimer, where for a monomer having a sedimentation coefficient of 7.4 S the two polymers would be expected to have sedimentation coefficients of 11.7 S and 15 S. These results may be compared with those of Brox and Hampton [1], who reported that at 4°C two components of 5.6 S and 8.8 S were observed in sucrose gradient centrifugation experiments in the presence of the same buffer.

Frontal analysis of enzyme on Sephadex G200

As the purified enzyme was to be used for a detailed kinetic study of the reaction mechanism [2,3], it was important to establish that, under the chosen conditions, only a single molecular species of enzyme was present or that different species exhibited virtually the same kinetic parameters. The results of initial velocity experiments with IMP, NAD and K^+ eliminated the possibility that multiple enzyme species with significantly different kinetic parameters were present. Thus the reaction conformed to Michaelis-Menten kinetics as judged by the fact that double reciprocal plots of reaction velocity as a function of reactant concentration were linear [2]. Frontal analysis on Sephadex G200, using the enzyme at a concentration comparable to those used for kinetic studies, showed that a single enzyme species is responsible for most, if

not all, of the catalytic activity (Fig. 2). In the profile obtained immediately after dilution of the enzyme there is a suggestion of a small irregularity on the leading side of the profile close to the plateau region, which could be caused by a minor component, but this is not observed on the trailing side of the profile. However, it will be noted that a change in the elution profile occurs when the diluted enzyme is applied to the column after standing for 20 h at 20°C. An increase in the time of standing up to 44 h did not cause any further change in the elution profile.

K_{av} values of 0.40 and 0.21 were calculated for the fresh and aged preparations, respectively. Values of K_{av} for a number of other proteins on Sephadex G200 were calculated from the data given by Andrews [5] and used to determine the weight average molecular weights of approx. 86 000 and 224 000 for the species applied to the column immediately and 20 h after dilution, respectively. What appears to be an increase in the weight average molecular weight on standing after dilution is consistent with polymerization or possibly with extensive unfolding of the enzyme molecule. The latter possibility is considered less likely because the total activity of the enzyme was not significantly reduced. While a polymerization would not be expected

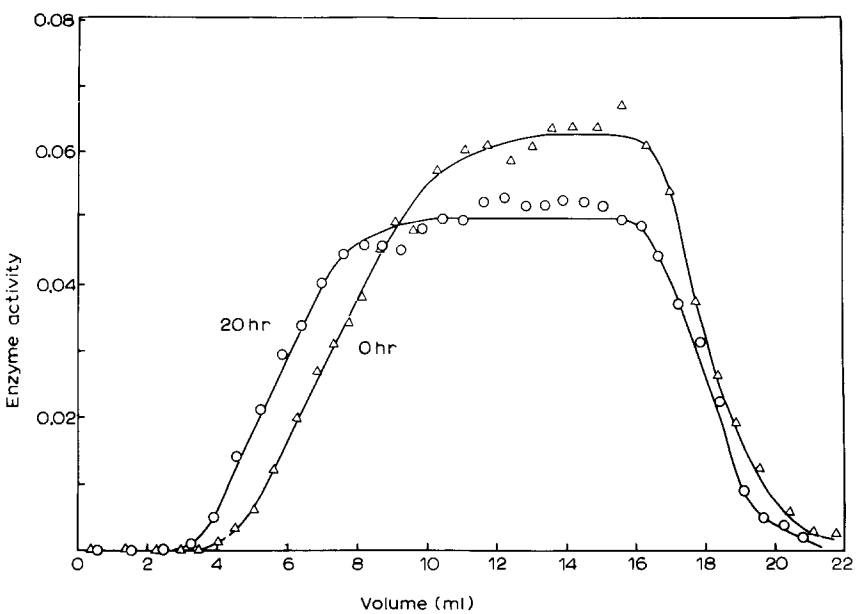

Fig. 2. Frontal analysis of IMP dehydrogenase immediately after dilution and after 20 h. The column (0.9 × 21.7 cm) contained Sephadex G200 in 0.1 M Tris · HCl buffer (pH 8.1) with 5 mM dithiothreitol. A stock enzyme solution of 3 mg/ml, stored at −15°C in 0.02 M Tris · HCl buffer (pH 8.1), was diluted to 40 μg/ml at 20°C in 0.1 M Tris · HCl buffer (pH 8.1) containing 5 mM dithiothreitol. Fractions were collected and assayed as described under Methods, the last fraction being collected 2.5 h and assayed 5 h after the experiment was begun. Reaction velocity was proportional to enzyme concentration over the range of concentrations occurring in the fractions. Activity on the vertical ordinate is expressed as μmol of NADH produced per min per ml of effluent. The activity of the applied sample of freshly diluted enzyme was 0.072 μmol NADH produced per min per ml. This fell slightly during the 5 h experiment to a level consistent with the plateau region (△) on the graph. After 20 h the activity was 0.053 μmol per min per ml, giving the applied activity for the second experiment (○).

to occur as a result of dilution, it could well be caused by the increase in temperature from the storage condition (−15°C) to room temperature (20°C).

Amino acid analysis and peptide mapping

The amino acid composition and peptide map of IMP dehydrogenase were used to assess its molecular size. The tryptic peptide map prepared from the enzyme showed approximately 40 peptides. The FITMOL computer program of Gibbs and McIntyre [9] was used to evaluate the fit of the amino acid composition to molecular sizes in the range corresponding to 30—50 tryptic peptides. The amino acid composition given in Table I was considered to be the best fit, corresponding to a molecular weight of 38 000 and 44 tryptic peptides. Two polypeptide chains of this size are reasonably consistent with a molecular weight of 86 000 from the results of frontal analysis and of 90 000 from sucrose gradient centrifugation [1].

Kinetic comparison of the two enzyme species observed on frontal analysis

A comparison was made of the kinetic properties of the species present initially (molecular weight approximately 86 000) with those of the species that forms on standing at 20°C. The results of initial velocity experiments for which IMP and NAD were varied in the presence of 5 mM dithiothreitol and 100 mM KCl show that the apparent kinetic constants for the two species are in reasonably good agreement (Table II). Hence no problems would be expected in a detailed kinetic study even if the enzyme is not totally in one or other of these forms.

TABLE I

AMINO ACID COMPOSITION OF IMP DEHYDROGENASE

The composition was determined with the FITMOL computer program of Gibbs and McIntyre [9]

Amino acid	Residues/mol (mol. wt. 38 000 [a])
Ala	35.1
Arg	24.8
Asp	26.9
Cys	3.2
Glu	41.1
Gly	38.1
His	6.9
Ile	22.0
Leu	29.9
Lys	18.5
Met	7.6
Phe	7.9
Pro	12.9
Ser	18.9
Thr	20.9
Tyr	6.2
Val	33.1

[a] No allowanee has been made for the tryptophan content.

TABLE II

KINETIC CONSTANTS FOR IMP DEHYDROGENASE

Experiments were performed in the presence of 0.1 M Tris · HCl buffer pH 8.1 containing the stated concentrations of dithiothreitol, 100 mM KCl, and varied concentrations of IMP (A) and NAD (B). The assay concentration of enzyme was 20 μg per ml. A typical set of data is illustrated in Fig. 1 (A, B) of the following paper [2]. The values for the apparent kinetic constants were obtained by computer fitting of each set of data as described in ref. 2.

Kinetic constant	Value of kinetic constant (mM)		
	Condition of enzyme before assay:		
	Freshly thawed 3 mg/ml	20 h at 20°C 40 μg/ml	Freshly thawed 3 mg/ml
	[Dithiothreitol] mM		
	5	5	0.1
	a	b	c
Apparent K_{ia}	0.030 ± 0.002	0.029 ± 0.004	0.052 ± 0.008
Apparent K_a	0.062 ± 0.004	0.055 ± 0.008	0.041 ± 0.010
Apparent $K_{ib} = \frac{K_{ia}K_b}{K_a}$	0.61 ± 0.06	0.48 ± 0.09	1.05 ± 0.32
Apparent K_b	1.08 ± 0.04	0.88 ± 0.07	0.84 ± 0.09

[a,b] Weighted mean values from 3 experiments. Values in Column a are from ref. 2.
[c] Values from a single experiment.

Activation of the enzyme by reducing agents

The enzyme was found to have an absolute requirement for a reducing agent in the assay mixture. The requirement could be met by a number of compounds whose effectiveness decreased in the order: dithiothreitol > mercaptoethanol > cyanide > ascorbate. The variation of reaction velocity with the concentration of dithiothreitol or mercaptoethanol is shown in Fig. 3. While at lower concentrations dithiothreitol is clearly a better activator than mercaptoethanol, it appears that the same maximum velocity is attained with each compound. On the other hand, the maximum velocities attainable with cyanide and ascorbate are only about 50% and 7%, respectively, of that attained with dithiothreitol. DL-Threitol does not activate the enzyme. At the lower concentrations of reducing agents there is a significant lag period after the initiation of the reaction by the addition of enzyme before the steady state velocity is attained. At concentrations of 0.02 and 5.0 mM, the times taken to reach the steady-state velocity were 3 and 1 min, respectively, for dithiothreitol and 6 and 2 min, respectively, for mercaptoethanol. The two phases of each plot in Fig. 3 suggest that the reducing agents break two types of disulphide bond which have different redox potentials. It appears that reduction of one type of bond produces active enzyme whose kinetic parameters differ from those of the species obtained on reduction of both types of bond (Table II). The two curves of Fig. 3 differ because of the difference between the redox potentials for mercaptoethanol and dithiothreitol.

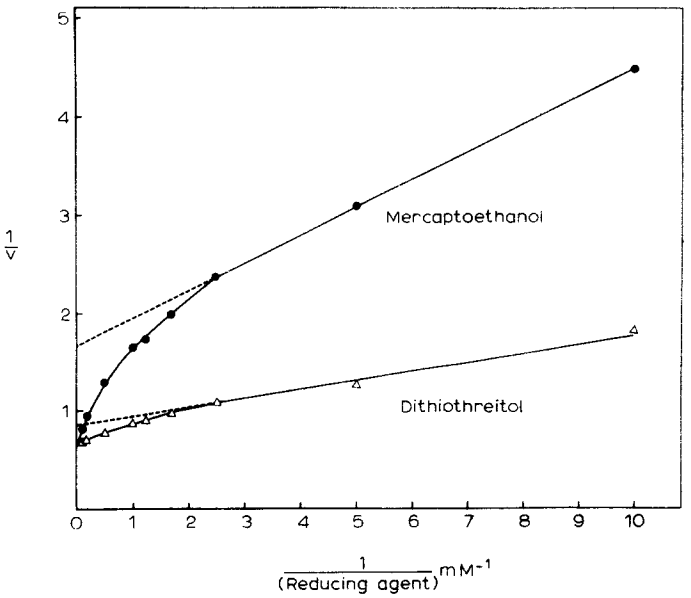

Fig. 3. Effect of dithiothreitol and mercaptoethanol concentrations on the steady-state velocity of the reaction catalyzed by IMP dehydrogenase. Assays were carried out under standard conditions as described under Methods, except that the concentration of reducing agent, dithiothreitol (△) or mercaptoethanol (○), was varied. Velocities are expressed as μmol of $NADH_2$ produced per min per mg protein.

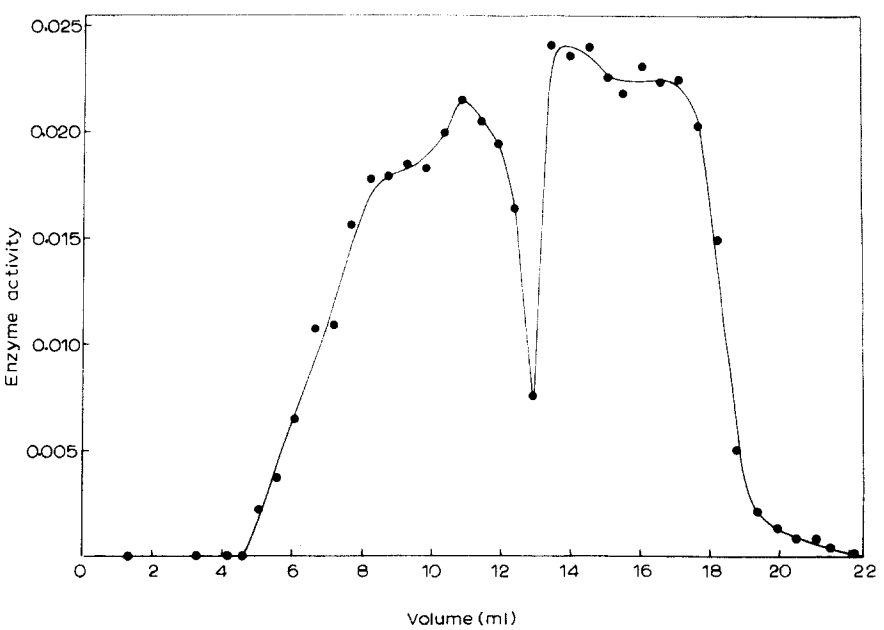

Fig. 4. Differential chromatography of IMP dehydrogenase. The column (0.9 × 22.6 cm) contained Sephadex G200 in 0.1 M Tris · HCl buffer (pH 8.1) without dithiothreitol. Enzyme was diluted from a stock solution (4.5 mg/ml in 0.02 M Tris · HCl buffer (pH 8.1)) to 20 μg/ml in 0.1 M Tris · HCl buffer (pH 8.1) containing 5 mM dithiothreitol. A column volume (14.4 ml) of the diluted enzyme was applied to the column and then eluted with 0.1 M Tris · HCl buffer (pH 8.1) containing 5 mM dithiothreitol. Fractions were collected and assayed as described under Methods. Activity was measured in the presence of 5 mM dithiothreitol as described under Materials and Methods and is expressed on the vertical ordinate as μmol of $NADH_2$ produced per min per ml of effluent. The activity of the applied material was 0.0218 μmol of $NADH_2$ produced per min per ml.

The occurrence of a change in the molecular species present as a function of dithiothreitol concentration is clearly demonstrated by differential chromatography [10] in which the enzyme was allowed to move from an environment containing 5 mM dithiothreitol to an environment lacking dithiothreitol. It is evident that the profile of enzyme activity has two distinct sections (Fig. 4). In the initial section the level of activity is lower than that applied, while in the second section the level is approximately that applied. The difference between the two levels reflects the faster migration rate (smaller elution volume) of the enzyme in the environment without dithiothreitol. This implies that the protein acquires a faster migration rate (larger weight average molecular weight) on passing through the junction between the environments with and without dithiothreitol, i.e. the enzyme polymerizes in the absence of dithiothreitol. The result obtained is unusual in that a minimum occurs in the middle of the profile, together with maxima immediately on either side. The position of the minimum, at approx. 13 ml elution volume, would correspond to the beginning of the elution of dithiothreitol which would be expected to elute at the total column volume (14.4 ml). The disturbance of the profile in this region may indicate a kinetically controlled system, with equilibration between dissociated and associated forms occurring at a rate comparable with the time taken for the separation of the species [11]. The molecular weight of the larger species has been calculated from the V_e to be 248 000.

Discussion

It is clear that a preparation of IMP dehydrogenase consists of a mixture of molecular weight isomers, and that the equilibria involved are affected in the direction of dissociation by increasing ionic strength [1], urea, sodium dodecyl sulphate or dithiothreitol. The effect of ionic strength indicates that ionic bonding is involved in the polymerization. The observation that increasing temperature causes polymerization of enzyme in dilute solution suggests that hydrophobic bonding could also be involved.

There is a variety of evidence to support the view that the kinetic properties of the enzyme are not dependent on its state of aggregation before assay. Thus each of the multiple protein bands obtained on gel electrophoresis exhibits enzymic activity and the values of the kinetic parameters are not significantly altered by the polymerization that occurs when dilute solutions of the enzyme, containing 5 mM dithiothreitol, are allowed to stand at room temperature (Table II). A further important observation is that the dialyzed enzyme, following exposure to 3 M urea, recovers virtually all its enzymic activity although the protein pattern on gel electrophoresis differs from that obtained with untreated enzyme. On the other hand, the change which occurs on lowering the concentration of dithiothreitol from 5 mM to 0.1 mM must differ from that referred to above as judged by the changes that occur in the values for the kinetic parameters (Table II).

The degree of dependence of IMP dehydrogenase activity on thiol compounds varies with the source of the enzyme and even with different preparations of the enzyme from the same organism. Thus the enzyme from Sarcoma 180 cells appeared not to require a thiol compound [12], that from *B.*

subtilis showed 70% of maximum activity in the absence of thiols [13], and that from *A. aerogenes* (the source for the present study) was reported by Magasanik et al. [14] to be usually stimulated 3 fold, although some preparations were completely inactive without a thiol compound. In the present work all preparations were found to be completely dependent on such an activator.

It is unusual for an enzyme to require for maximum activity dithiothreitol in the region of 5—10 mM. This requirement cannot be account for by dithiothreitol functioning as a substrate because the amount of $NADH_2$ formed during a reaction is not limited by the amount of reducing agent present. Rather does it appear that dithiothreitol activates by reducing two different types of disulphide bond in an inactive polymer to form, over a period of a few minutes, active enzyme species of lower molecular weight. The effect of reducing agents on the state of polymerization of the enzyme may have implications with respect to its intracellular activity.

Acknowledgements

We thank Dr D.C. Shaw for amino acid analysis and peptide mapping, Dr A.J. Gibbs for the computer analysis to assess the size of the protein from its composition, and Professor L.W. Nichol and Dr P.D. Jeffrey for performing the sedimentation velocity experiment and for helpful discussion. Thanks are also due to Professor W.W. Cleland for his comments on the manuscript.

References

1 Brox, L.W. and Hampton, A. (1970) Biochim. Biophys. Acta 206, 215—223
2 Heyde, E., Nagabhushanam, A., Vonarx, M. and Morrison, J.F. (1976) Biochim. Biophys. Acta 429, 645—660
3 Heyde, E. and Morrison, J.F. (1976) Biochim. Biophys. Acta 429, 661—671
4 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
5 Andrews, P. (1970) Methods Biochem Anal. 18, 1—53
6 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
7 Shapiro, A.L., Vinuela, E. and Maizel, Jr., J.V. (1961) Biochem. Biophys. Res. Commun. 28, 815—820
8 Hedrick, J.L. and Smith, A.J. (1968) Arch. Biochem. Biophys. 126, 155—164
9 Gibbs, A.J. and McIntyre, G.A. (1970) J. Gen. Virol. 9, 51—67
10 Baghurst, P.A., Nichol, L.W., Richards, R.J. and Winzor, D.J. (1971) Nature 234, 299—301
11 Nichol, L.W. (1964) Aust. J. Sci. 27, 342—348
12 Anderson, J.H. and Sartorelli, A.C. (1968) J. Biol. Chem. 243, 4762—4768
13 Yokosawa, H., Tobita, T. and Yamada, T. (1971) Biochim. Biophys. Acta 227, 538—553
14 Magasanik, B., Moyed, H.S. and Gehring, L.B. (1957) J. Biol. Chem. 226, 339—350