Metabolic Control and Structure of Glycolytic Enzymes. IV. Nicotinamide–Adenine Dinucleotide Dependent *in Vitro* Reversal of Dissociation and Possible *in Vivo* Control of Yeast Glyceraldehyde 3-Phosphate Dehydrogenase Synthesis*

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ABSTRACT: A systematic study of the variables affecting the *in vitro* reassembly of yeast glyceraldehyde 3-phosphate dehydrogenase from urea-dissociated subunits led to the following optimum reversal conditions: (1) pH 6.9, 0.05 M imidazole buffer; (2) 0.3 M KCl; (3) 0.05 mg/ml of protein concentration; (4) 27 mM nicotinamide–adenine dinucleotide; (5) 27 mM glutathione; and (6) temperature: dilution at 0°, followed by incubation at 16° for 1 hr. The refolding of yeast D-glyceraldehyde 3-phosphate dehydrogenase exhibits an almost absolute requirement for nicotinamide–adenine dinucleotide. In 0.15 M KCl, to approximate *in vivo* conditions, the recoveries of activity relative to the native enzyme control are 3 and 82% respectively; with 0.8 M KCl, it is 93%. The profile of activity recovery shows a low activity plateau at nicotinamide–adenine dinucleotide concentrations below 10^{-5} M, an ionic strength dependent transition centered in the region 0.6–2.2 mM, and a high activity plateau above 27 mM. High salt (0.8 M KCl) substitutes for nicotinamide–adenine dinucleotide, yielding 68% recovery. The 3-acetylpyridine nicotinamide–adenine dinucleotide analog serves as a weak coenzyme and enhances the refolding whereas nicotinamide–adenine dinucleotide phosphate does neither; therefore, a free adenosyl 2′-hydroxyl appears to be critical, but the nicotinamide–adenine dinucleotide pyridineamide probably is not. The *in vitro* reassembly is fast (half-time = 7 min at 16°) and gives complete recovery (93%) suggesting that it approximates the *in vivo* folding process. The *in vitro* requirement for nicotinamide–adenine dinucleotide for folding of yeast D-glyceraldehyde 3-phosphate dehydrogenase emphasizes that the amino acid sequence alone may not be adequate to provide the correct folding of nascent or completed polypeptides *in vivo* and that nicotinamide–adenine dinucleotide may accomplish this and thereby control the rate of enzyme synthesis. Two models are described for translational control by control of folding. The procedures used here gave activity recoveries greater than 50% with six different enzymes.

One major objective of biochemistry is the *in vitro* assembly of complex cellular structures from a disorganized mixture of the pure, biochemical cellular components, such as enzyme and structural protein polypeptide chains. The accomplishment of this *in vitro* will require a detailed understanding of the effects of various conditions upon the assembly of polypeptide chains to active enzymes and other specific structures.

A second major objective of biochemistry is to achieve a knowledge of the control of metabolism and other cellular functions, in terms of the structure of the biochemical components, especially enzymes, which control or carry out the functions. This control includes, among other things, regulation of enzyme levels through the control of the rate of synthesis and degradation of enzymes. There is a need to elucidate mechanisms to explain control of enzyme synthesis at the level of translation. It is clear that in a great many different forms of life, involving both differentiating and mature cells, primary control of protein synthesis is exerted at the level of translation, through a whole range of mechanisms (for reviews, see Gross, 1968, and Vogel and Vogel, 1967).

Since the forces which control the *in vitro* rate of enzyme synthesis may be closely related to the forces which affect the *in vitro* assembly of polypeptide chains, there is a strong connecting link between the two previously mentioned objectives. The following questions have a bearing upon both objectives. (1) "What governs the *in vivo* folding and assembly of nascent polypeptide chains into active enzymes or other specific protein structures?" (2) "What effects, if any, do the metabolites[1] or constituents of the cell have upon these processes?" (3) "What is the significance, in terms of control, of any effects of metabolites on the folding and assembly of nascent polypeptide chains?"

As part of a systematic study of the reassembly of glycolytic enzymes from their unfolded polypeptide chains (Deal *et al.*, 1963a,b; Johnson *et al.*, 1969; Holleman and Deal, 1969; S. P. Blatti and W. C. Deal, in preparation), this paper reports a study of the requirements for reassembly of yeast glyceraldehyde 3-phosphate dehydrogenase. A similar systematic study of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase is reported elsewhere (Deal and Constantinides, 1967; S. M. Constantinides and W. C. Deal, in preparation).

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[1] Metabolite is used here in its broadest sense, to mean any cellular constituent which can be chemically altered.

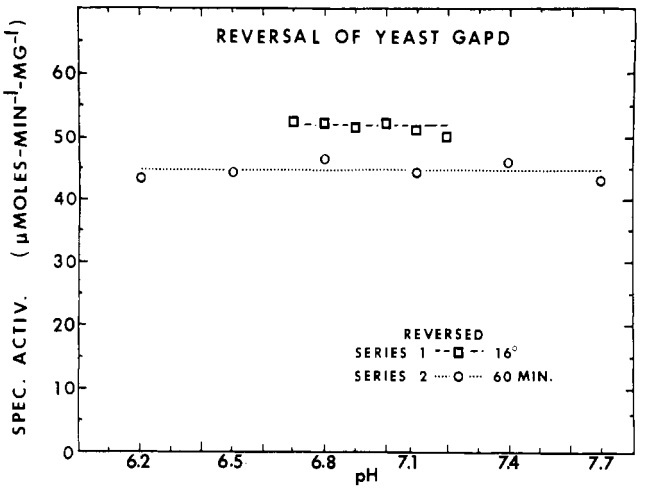

FIGURE 1: Effect of pH. The two experiments were run completely separately, but the experimental procedures were identical. Both were exposed to the 8 M urea dissociation mixture at 0° for 4 hr; then reversal of dissociation was accomplished by dilution into the reversal solvents at 0° at the various pH values, followed by incubation at 16°. The optimum refolding mixture was used with the exception that 0.1 M imidazole buffers with various pH values between 6.2 and 7.7 were prepared as stock solutions. (See Methods for dissociation and refolding solvents and procedures.)

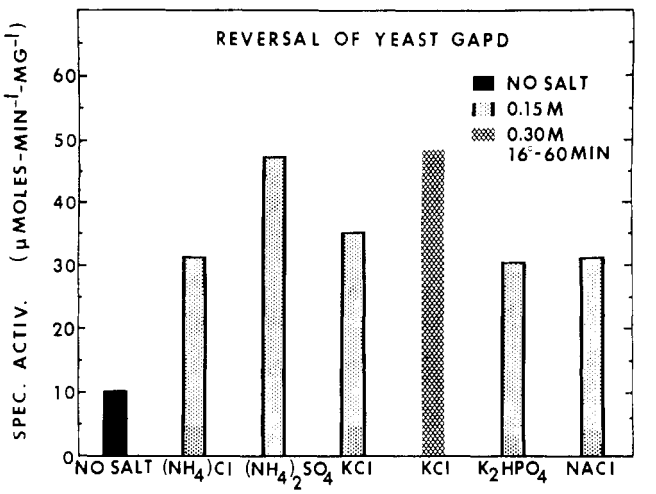

FIGURE 2: Effect of salt species. Samples were exposed to the dissociation mixture 5 hr, before refolding as in the legend of Figure 1. The optimum refolding mixture was used except that for each 1.0 ml of reversal solvent, 0.25-ml volume was distributed appropriately between a salt stock solution and water to give the final salt concentrations.

The reassembly of yeast glyceraldehyde 3-phosphate dehydrogenase from its unfolded polypeptide chains has been found to require[2] the cofactor NAD, which explains the failure of earlier workers to obtain reversal of dissociation with this enzyme (Chilson *et al.*, 1966). The yeast glyceraldehyde 3-phosphate dehydrogenase–NAD reassembly system also has several unique characteristics which have not been observed in previous reassembly studies with other enzymes: (1) the metabolite required for reassembly is not permanently bound to the enzyme as part of the folded enzyme product; and (2) the metabolite is required throughout the reassembly process; it cannot be added after a partial refolding in the absence of metabolite.

Consideration of the significance of the *in vitro* requirement for NAD for folding of yeast glyceraldehyde 3-phosphate dehydrogenase has led to preliminary models in which these effects could control enzyme synthesis through control of enzyme folding (Deal, 1967; Deal and Constantinides, 1967). Two models are described in this paper in which control of enzyme synthesis may be exerted at the level of translation through control of folding. Both models invoke a mechanism of product inhibition of the completion of synthesis of incomplete nascent polypeptide chains. *The yeast glyceraldehyde 3-phosphate dehydrogenase–NAD refolding system is the first where a metabolite with a potential for enzyme synthesis control has actually been demonstrated to be capable of controlling the folding of the enzyme.*

Results

The optimum conditions for refolding were found by using a successive approximation type of approach. The results presented here are from the final analyses, in which all variables save that being tested were at their optimum values.

Effect of pH and Salt Species. In preliminary experiments in which not all variables were optimized, a marked pH optimum near pH 6.9 was observed. However, in experiments in which all other variables were optimum, the pH profile of the recovery of activity upon reversal showed no dependence upon pH in the range of pH 6.2–7.9 (Figure 1).

Figure 2 shows that the three monovalent salts, ammonium chloride, potassium chloride, and sodium chloride, yielded essentially identical recoveries in the 0.15 M concentration tests. At the same concentration, potassium phosphate gave essentially the same recovery as the monovalent salts, but ammonium sulfate produced substantially greater recovery. However, the 0.3 M KCl sample showed a recovery equal to the 0.15 M ammonium sulfate sample. This suggested that this might be only a general ionic strength and not a salt-specific effect, despite the fact that potassium phosphate did not fit the general pattern.

Figure 3 shows data for the reversed enzyme and the native control for KCl concentrations ranging up to 1.0 M. There was a marked enhancement of the reversal by KCl but almost no effect upon the native enzyme. In the reversal samples, the specific activity at 0 M KCl concentration was only about 40, but this doubled to about 85 with 0.12 M KCl and was almost four times as great at 0.3–0.5 M KCl, where it appeared to achieve a maximum. This was a rather surprisingly high optimum ionic strength. We chose the lower concentration, 0.3 M, as our optimum salt concentration, on the basis that this was nearer the value expected *in vivo* than was 0.5 M. Later studies on the NAD effects at various ionic strengths confirmed that 0.3 M gave as good recovery (92%) as 0.5 M KCl. In the ammonium sulfate system, the optimum recovery of activity occurred at an ionic strength of 0.42. Since KCl yielded recoveries as high as those with ammonium sulfate

[2] This means that the metabolite is necessary for attainment of a "reasonable" rate of folding; *i.e.*, in its absence, the rate of correct folding is extremely slow. The metabolite may, or may not be absolutely essential for correctly formed enzyme.

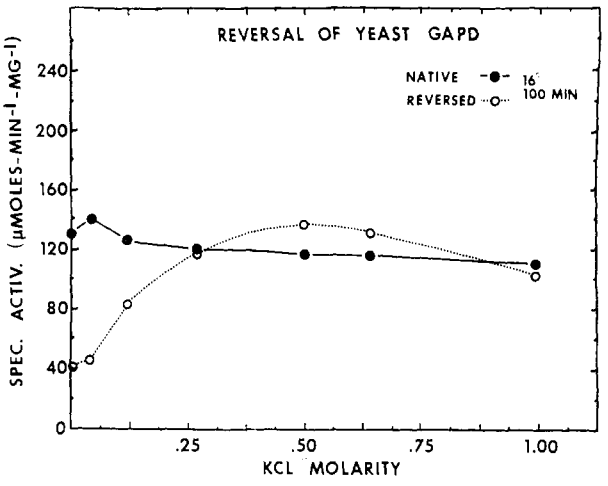

FIGURE 3: Effect of KCl. Test samples at 5 mg/ml were exposed to the urea dissociation solvent at 0° for 3 hr while the native enzyme control samples were treated identically except that the "control dissociation solvent" had no urea (appropriate water was added in its preparation to produce the usual concentration of the components other than urea; see Methods). A partial stock refolding solution was made by mixing 50, 10, and 15 volumes, respectively, of 0.1 M imidazole·HCl (pH 6.9), 0.027 M sodium glutathione (pH 6.9), and 0.183 M NAD (pH 6). Then 1.0-ml refolding solutions at various salt concentrations were prepared by addition of appropriate volumes of 4 M KCl and water to 0.75 ml of the partial stock above.

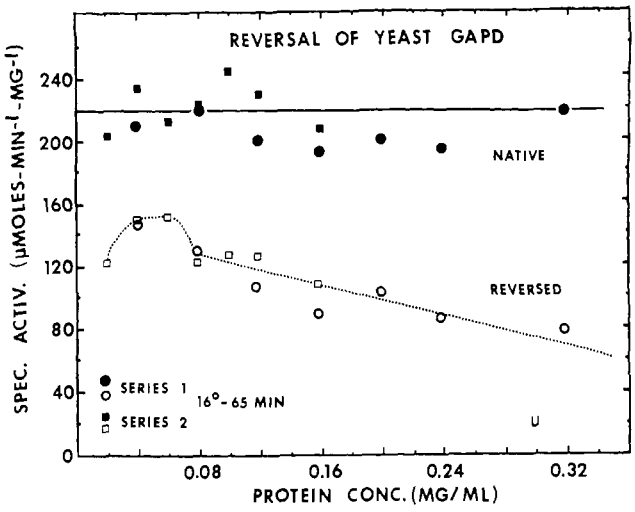

FIGURE 4: Effect of protein concentration. Samples were exposed to the urea dissociation system for 3.5 hr (series 1) and 5 hr (series 2), respectively. Refolding was carried out using the optimum refolding solvent as described in Methods, but parts of the dissociation and refolding procedures were adapted to the specific requirements of this experiment (see text).

and was more convenient to use, it was selected as the salt for the optimal refolding mixture.[3]

Effect of Protein Concentration. Two series of experiments were performed, using two separate stock samples, 32 and 16 mg per ml. Data from typical experiments on activity recovery as a function of protein concentration in the refolding solution are shown in Figure 4. The refolding appeared to be enhanced by decreasing concentration in the region from 0.32 to *ca.* 0.04–0.06 mg per ml, at which point it seemed to go through a maximum. It should also be noted that the enzyme concentration in the urea dissociation mixture was not a major factor under these conditions. The two sets of experiments agreed quite well at the low concentrations, and fairly well at the higher concentrations.

The increasing recovery with decreasing concentration down to 0.04–0.06 mg/ml suggested that steric effects might have been causing nonspecific aggregation at higher concentrations. On the other hand, the decrease in recovery with decreasing concentration below about 0.04 mg/ml suggested a requirement for a minimum concentration for the reassembly process. The kinetic experiments in the next section provided further evidence that the reversal process was concentration dependent.

Concentration Dependence of the Half-Times of the Refolding Process at 16°. If the requirement for a minimum concentration reflected a requirement for polymerization for activity, then the half-time of the process should be greater at lower concentrations than at higher concentrations. On the other hand, if the subunits were active in their monomer form, but were being inactivated, for example, by adsorption onto the glass walls of the reversal tube, then the half-times of the reaction should be the same at all concentrations. To distinguish between these possibilities, the recovery of activity was analyzed as a function of time at 16° at two greatly different protein concentrations, 95 and 5 μg per ml.

As shown in Figure 5, the half-time at a protein concen-

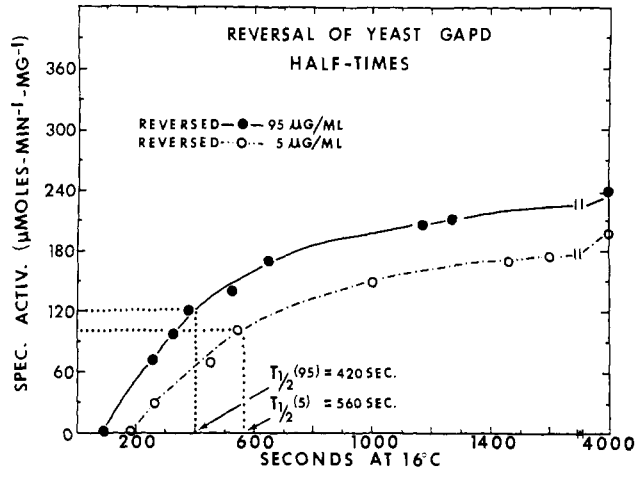

FIGURE 5: Half-times at 16° at 95 and 5 μg per ml. The study of the two concentrations was made three separate times, after 2, 5, and 7 hr in urea. The data shown are typical of the results. The dissociated enzyme (9.5 mg/ml) was exposed to the urea for 2 hr. The standard optimum refolding mixture was modified slightly. To begin the experiment, a urea enzyme sample was diluted at 0°, shaken, and taken to 16°. For refolding at 95 μg/ml, 10 μl of urea dissociated enzyme was diluted into 1.0 ml of the reversal mixture at 0°; for the reversal at (5 μg/ml), 1 μl of urea-dissociated enzyme was diluted into 2 ml of the refolding mixture of 0°. The time of addition of the enzyme into the assay, which presumably stopped reassociation, was chosen as the reference time.

[3] The same relative effects appear to be observed, independent of the specific activity of the starting enzyme. The remaining data to be shown are from experiments which were repeated with enzyme of high specific activity to ensure that this was not an important factor.

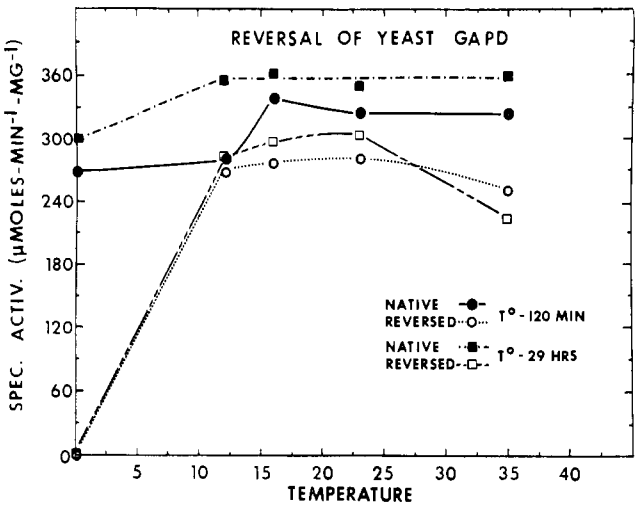

FIGURE 6: Effect of temperature. The samples were exposed to the urea dissociation mixture for 3 hr at 0°. The optimum refolding mixture was used. Samples were diluted at 0°, as described in Methods, and then exposed to the various temperatures for the indicated time (see text).

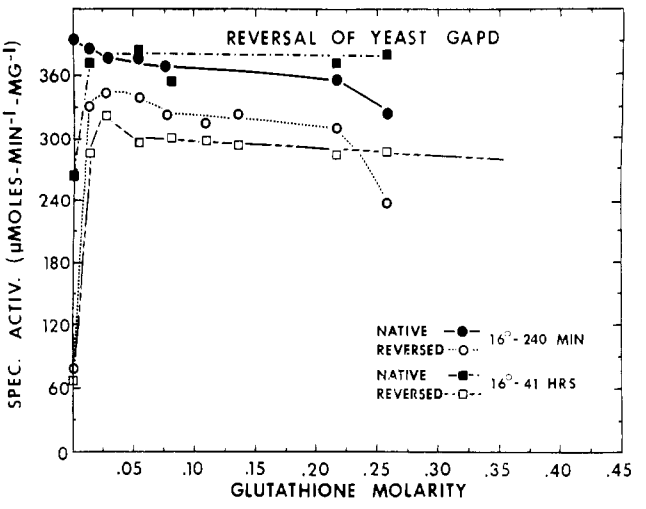

FIGURE 7: Effect of glutathione. Samples were exposed to the urea dissociation mixture for 5 hr at 0°. Reversal was accomplished as described in Figures 1 and 2 and Methods. Various glutathione–water stock solutions were prepared by mixing water with stock glutathione (0.54 M, pH 6.9).

tration of 95 μg/ml was 420 sec, while that at 5 μg/ml was distinctly longer, 560 sec. This experiment was repeated three times and the results were essentially identical. These differences appeared to show conclusively that the recovery of activity was a concentration-dependent process. However, the reaction appeared to be mixed order since, for a second-order process, the ratio of the half-times at two concentrations should be inversely proportional to the two concentrations. This was not observed here.

Effect of Temperature. As seen in Figure 6, after refolding 120 min in 0.3 M KCl, the temperature profile of activity showed a fairly broad maximum in the range 12–35°; but the highest recovery was obtained with the 16° and 23° samples, which had approximately equal recovery. Because it was the lowest temperature that gave maximal activity, 16° was selected as the temperature for the standard optimal reversal conditions. An additional favorable characteristic was that it also seemed to show less destabilization over long periods of time than that observed at higher temperatures (see the 29-hr curves in Figure 6).

Time at 0°. The activity recovery at 16° was described in the previous section on concentration dependence of the half-times for reassembly. It was also of interest to evaluate the effect of time of incubation at 0°, which preceded the 16° incubation.

Experiments were conducted in which the refolding samples were kept at 0° for varying times, subsequently incubated at 16° for 1 hr, and then assayed. There was no change in activity with time of incubation at 0° for at least as long as 2 hr, in any of these systems.

Effect of Glutathione Concentration. The presence of a very low concentration of glutathione was adequate to yield excellent recovery of activity upon refolding. The sample with 0.013 M glutathione had substantial activity (Figure 7). There was a broad optimum at 0.027 M, which was confirmed by repeated experiments. Above this concentration, however, there seemed to be slightly less recovery, although the recovery was still quite high. The native control was similarly affected. Similar destabilization at high concentrations of reducing agents was seen in mercaptoethanol; furthermore, separate stability studies (G. M. Stancel and W. C. Deal, Jr., 1967, unpublished results) suggested that the enzyme experienced significant structural change under these conditions.

The long-term (41-hr) exposure sample showed a similar activity profile, with an optimum at 0.027 M and a plateau in the range 0.05–0.26 M; however, neither the refolded nor the native samples at 0.26 M showed the apparent destabilization obtained in the short-term study. Presumably the decrease in reducing agent due to air oxidation accounted for this. After a 5-day exposure, no activity was obtained in any of the native or refolded samples with glutathione concentrations less than 0.054 M. In contrast, the sample refolded in 0.081 M glutathione retained about 75% of its original activity and the 0.1–0.26 M samples retained about 90% of their original activity. Similar results were obtained for the native enzyme samples. None of the samples, native or refolded, had activity after 11 days at 16°. Mercaptoethanol gave generally similar results, although differing in some respects.

Requirement for NAD for Refolding. The initial attempts to achieve reassembly were unsuccessful until NAD and the substrate, D-glyceraldehyde 3-phosphate, were tested as components of the reassembly mixture. It is very significant that NAD had to be present in the reassembly mixture *when it was brought to 16°* to start the folding process; addition of the NAD after previous incubation at 16° yielded no activity recovery. Subsequent analysis revealed that D-glyceraldehyde 3-phosphate had little or no effect on reversal and also that the NAD effect was dependent upon the ionic strength.

Figure 8 shows the pronounced effect of NAD concentration upon reversal in the range of 0–54 mM NAD. The effect was studied at four different KCl concentrations, 0.15, 0.3, 0.5, and 0.8 M. For 0.15 M KCl, the specific activity profile of the reversed enzyme samples showed: (1) a very low activity plateau of about 12 at NAD concentrations below 10^{-5} M, (2) a transition centered about the region of 2.2 mM NAD K_F (see below), and (3) a very high specific activity plateau of

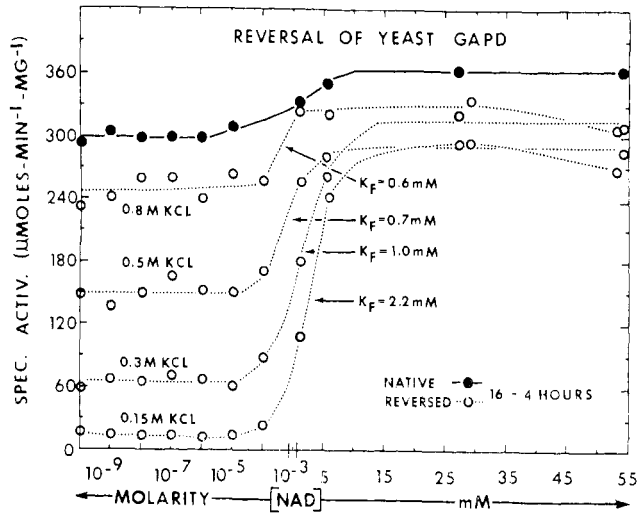

FIGURE 8: Effect of NAD. The experiments at the four KCl concentrations were done in pairs, 0.15 and 0.80 M together, and 0.3 and 0.5 M together. Although performed separately, both pairs were exposed to the urea-dissociating system for 3 hr. For simplicity, only the control run in 0.3 M KCl is shown; the others were practically identical with it. For the 0.15 and 0.80 M KCl pair, a 0.4 M imidazole buffer (pH 6.9) was prepared and 0.125 ml was added per final 1.0 ml of reversal mixture. Within the pair, for the 0.15 M KCl solvent, 0.075 ml of 2 M KCl was added and for the 0.8 M reversal solvent, 0.40 ml of 2 M KCl was added. The water volumes were 0.55 and 0.225 ml, respectively. For the 0.3 and 0.5 M samples, 0.5 ml of the standard 0.1 M imidazole buffer was added, then 0.15 and 0.25 ml of 2 M KCl, respectively. The water volumes added were 0.10 ml and none, respectively. A 0.4 M NAD stock solution was prepared and titrated to pH 6 with 2 N NaOH, yielding 0.33 M NAD. Additional NAD stock solutions were prepared by diluting portions of this "main" stock. Then 0.15 ml of the appropriate NAD stock was added to the refolding mixture tubes which, with all additions, attained a final volume of 1.0 ml.

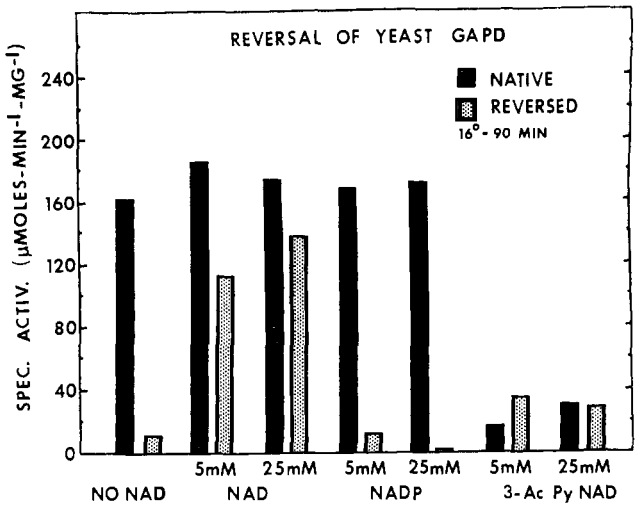

FIGURE 9: Effect of NAD analogs. The test samples were exposed to the urea dissociation conditions for 4 hr at 0°. A partial stock refolding solution was made by mixing 0.1 M imidazole·HCl (pH 6.9), 0.27 M glutathione (pH 6.9), and 2 M KCl in volumes of 50, 10, and 15, respectively. Then nucleotide solutions were prepared by making 0.2 M nucleotide stock solutions, titrating these to pH 6.9, and diluting appropriately with water so that addition of 0.25 ml of stock nucleotide to 0.75 ml of the partial stock solution would yield the 5 or 25 mM solution desired.

about 300 at NAD concentrations equal to, or greater than 27 mM. Thus, the activity recovery at low NAD, or in its absence was only 4% of that obtained in the presence of 27 mM NAD, with this KCl concentration of 0.15 M. At high NAD concentrations the activity recovery was 82%. Recoveries up to 93% were obtained at higher ionic strengths.

We have designated the NAD concentration at which the half-maximum enhancement occurred as K_F, the folding constant, by analogy to the Michaelis constant in catalysis. This presumably provides a fair measure of the tightness of binding of NAD at the enzyme site responsible for the reversal enhancement. As shown in the next section, the K_F decreased markedly with increased ionic strength.

The data for 0.3, 0.5, and 0.8 M KCl in Figure 8 show that the activity recovery with low or zero NAD concentrations increased strongly with increasing ionic strength; the specific activity of enzyme refolded in the absence of NAD increased from 12 in 0.15 M KCl to 245 in 0.80 M KCl. In addition, the NAD K_F was shifted to lower values with increasing ionic strength, going from 2.2 mM NAD at 0.15 M KCl to 0.6 mM at 0.80 M KCl, suggesting that NAD was bound more tightly and consequently, a lower NAD concentration was able to saturate the NAD binding site which enhanced folding. Concomitant with the decrease in K_F with increase in ionic strength, the difference in per cent recovery between refolding with 27 mM NAD and that with no NAD decreased from 79% with 0.15 M KCl to only 25% with 0.8 M KCl. It thus appeared that the high concentrations of KCl were able to effectively substitute for NAD in enhancing refolding.

Note finally that there did seem to be a small but significant activation of the native enzyme by NAD and that this process also seemed to be characterized by a binding constant similar to that for the refolding.

Long-term stability studies (40 hr at 16°) clearly confirmed the general results just described. A further important point shown by these stability tests was that the *native enzyme* was quite stable in the refolding mixture *without NAD*, for periods as long as 40 hr. There was some slight enhancement of stability (about 10–20%) with about 0.5–27 mM NAD, but the effect was too small to be used as an explanation for the NAD enhancement of refolding.

Effect of NAD Analogs upon Refolding. The possibility that the refolding enhancement by NAD might be the result of NAD binding at the catalytic site made it desirable to compare the structural requirements for refolding enhancement with the structural requirements for coenzyme catalytic function. Many of the latter properties are available from the extensive studies on the AcPyNAD[4] analog by Kaplan and coworkers (Kaplan and Ciotti, 1956; Kaplan *et al.*, 1956, 1957; Kaplan, 1960) and by Stockell (1959a,b).

Two structural analogs of NAD were tested for their ability to enhance the refolding. Figure 9 shows data for the native control enzyme and enzyme refolded with no added NAD, and in the presence of 5 or 25 mM NAD, NADP, and 3-AcPyNAD, respectively.

The NADP, which is not a substrate for the enzyme, did

[4] Abbreviation used is: 3-AcPyNAD, the oxidized form of the 3-acetylpyridine analog of NAD, in which the O=CNH_2 group attached to carbon 3 of the pyridine ring is replaced by O=CCH_3.

not enhance refolding at 5 mM and in fact, showed inhibition of the refolding process, but not of native enzyme activity, at 25 mM. Refolding in the latter system also resulted in some precipitation of the enzyme. Thus, these data suggested that a key structural requirement for enhancement of the reversal was the presence of a free 2′-OH on the adenosine moiety since addition of the 2′-phosphate on the ribose ring of adenosine in NADP prevented enhancement of the reversal. This is similar to the requirements for binding at the catalytic site (Yang and Deal, 1969a).

The 3-AcPyNAD analog is only a weak coenzyme for the catalytic reaction and it inhibits the activity of the enzyme assayed with NAD as a cofactor (Kaplan *et al.*, 1956). At 5 mM, where the native enzyme was strongly inhibited by 3-AcPyNAD, the recovery of activity upon reversal was significantly enhanced, being about threefold greater than that observed with no added pyridine nucleotide. At both 5 and 25 mM, the recovery of refolded enzyme was essentially the same as that of the native enzyme, raising the possibility that the refolding might have been essentially complete. However, the fact that the native enzyme was inhibited complicated the picture somewhat. The effects of 3-AcPyNAD and other analogs on the refolding are receiving further attention.

Comparison of Sedimentation Coefficients of Refolded and Native Enzyme. The sedimentation coefficients of the native and refolded enzymes were identical within experimental error. This apparent identity of gross structure, taken with the fact that essentially complete recovery of activity was obtained upon refolding offered moderately strong evidence that the refolded enzyme was identical with the original native enzyme.

Discussion

The Extent of the NAD Enhancement of in Vitro Reversal of Dissociation of Yeast Glyceraldehyde 3-Phosphate Dehydrogenase. The most striking discovery in this work was the NAD requirement for the *in vitro* reassembly of yeast glyceraldehyde 3-phosphate dehydrogenase from its subunits dissociated and unfolded by 8 M urea. Under the conditions of KCl concentration thought to most closely approximate those *in vitro*, namely, 0.15 M KCl, the *in vitro* reversal of the dissociation of yeast glyceraldehyde 3-phosphate dehydrogenase exhibits an almost total requirement for the presence of NAD. It shows a 96% increase in recovery in the presence of 27 mM NAD over that in its absence. Even allowing for an *in vivo* KCl concentration as high as 0.3 M, an increase of 80% would still be observed in the presence of 27 mM NAD, over that in its absence. (Only slightly less recovery would be obtained with 5 mM NAD.) It seems unlikely that, even in compartmentalized systems, the KCl concentration (or for that matter, the total ionic strength of all ions), would be much greater than 0.3 M. These results show that the amino acid sequence alone is not adequate to determine the folding of yeast glyceraldehyde 3-phosphate dehydrogenase.

The NAD concentration *in vivo* is probably always less than 0.5 mM, so that if the NAD effect on glyceraldehyde 3-phosphate dehydrogenase folding occurs in *vivo*, the rate of folding of yeast glyceraldehyde 3-phosphate dehydrogenase is probably less than the half-maximum rate (Figure 8). Furthermore, since this NAD concentration lies in the transition region of the curve (Figure 8), where a small change in NAD concentration gives rise to a large change in rate of folding of yeast glyceraldehyde 3-phosphate dehydrogenase, the rate of folding, and presumably the corresponding rate of synthesis (see next section), would be very sensitive to small changes in NAD concentration.

Two Models for Protein Synthesis Control at the Translation Level. There is overwhelming evidence that in many living systems, including both differentiating and mature cells, protein synthesis must be regulated during translation, the series of reactions where a polypeptide chain is produced from its mRNA template (for reviews, see Vogel and Vogel (1967) and Gross (1968)). It also seems clear that a whole range of different mechanisms operate to control translation. The results reported in this paper suggested one possible mechanism by which this might be accomplished.

The mechanism involves a "folding control" of translation by metabolites. This idea (Deal, 1967; Deal and Constantinides, 1967) in various forms has been considered earlier and rejected (Jacob and Monod, 1961) and also reproposed independently (Gruber and Campagne, 1965; Cline and Bock, 1966). The work reported here is unique in that it provides the first direct experimental evidence that a potentially regulatory metabolite actually is capable of exerting a "control of folding" of the polypeptide chains of its metabolically related enzyme.

Using the mechanism of control of translation by metabolite control of folding of polypeptide chains, two different models have been developed. Both invoke product inhibition of the completion of synthesis of nascent polypeptide chains at intermediate stages of completion. The first model best fits the yeast glyceraldehyde 3-phosphate dehydrogenase results. The second model does not fit the yeast glyceraldehyde 3-phosphate dehydrogenase results, but may apply to certain other proteins.

A. Model 1: Incomplete product inhibition (intrachain inhibition). This model makes three assumptions. (1) Much of the folding of a polypeptide chain into its globular conformation occurs during its synthesis while it is a nascent polypeptide chain; as a minimum, it undergoes at least one critical folding step at the nascent polypeptide chain level. (2) Unless the growing nascent polypeptide chain is folded "correctly" at each step of its growth process, it may inhibit the addition of amino acids to itself (intrachain inhibition). (3) There is a correspondence between the requirements for "correct" *in vitro* folding and assembly of complete, unfolded polypeptide chains and the "correct" *in vivo* folding and assembly of incomplete, nascent polypeptide chains as they are being synthesized. This assumption provides the basis for the experimental test of the model and mechanism.

Figure 10A,B illustrates schematically the structural relationships between components involved in protein biosynthesis and shows how improper folding might inhibit the process of addition of amino acids at the polypeptide chain growth site, using model 1. The A′B′C′ anticodon on the appropriate peptidyl-tRNA is shown complexed with the ABC codon on the mRNA. The figures represent two hypothetical possibilities in the synthesis of a polypeptide chain of yeast glyceraldehyde 3-phosphate dehydrogenase. The synthesis of the enzyme is assumed to proceed fairly extensively until a critical stage is reached. Beyond this, synthesis cannot proceed in the absence of NAD (Figure 10A) because the nascent polypeptide chain is folded incorrectly, thereby blocking the growth site. But if NAD is present, it can and does proceed

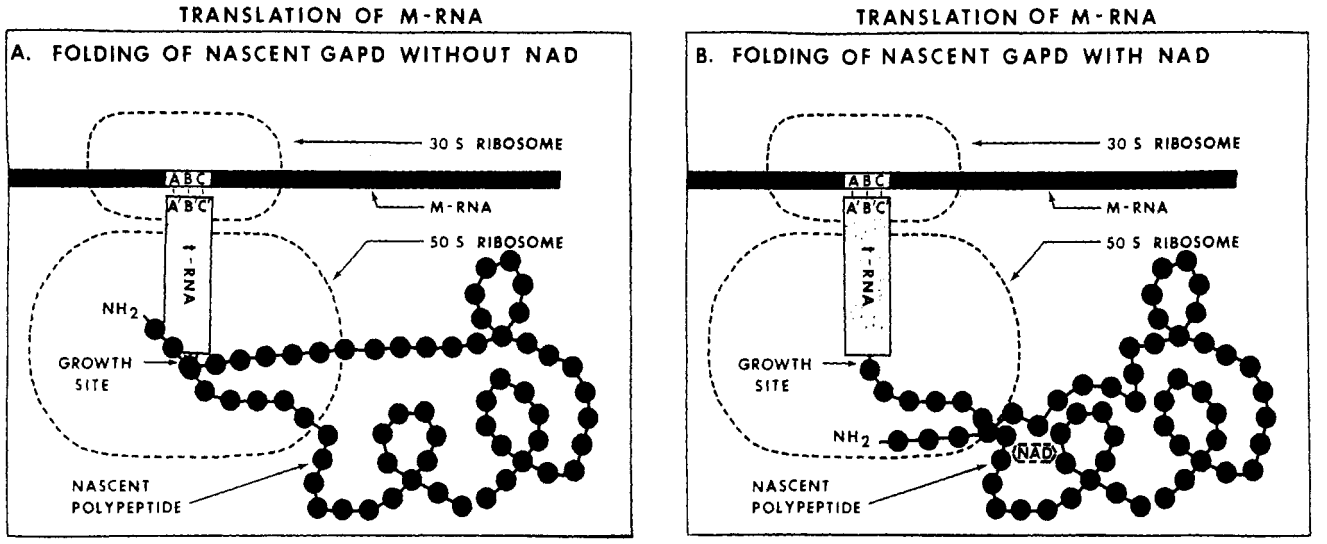

FIGURE 10: Model for NAD control of D-glyceraldehyde 3-phosphate dehydrogenase folding and synthesis. See text for details.

(Figure 10B), because the growth site is then cleared of the interfering segment of nascent polypeptide chain.

It should be noted that for model 1 to be effective in controlling synthesis of yeast glyceraldehyde 3-phosphate dehydrogenase, the requirement for NAD must manifest itself *during synthesis;* if it occurs *after synthesis,* polypeptide chains would be synthesized even in the absence of NAD, and this would be useless since they could not yield active enzyme without NAD (but see model 2, where a requirement for a metabolite for folding could occur *after synthesis* and *still* exert synthesis control).

B. MODEL 2. COMPLETED PRODUCT INHIBITION (INTERCHAIN INHIBITION). This model makes two assumptions. (1) There are no critical folding steps during the synthesis of the polypeptide chain and if no other factors influenced it, the polypeptide chain would be synthesized to completion in the absence of the metabolite. (2) Incorrectly folded, completed polypeptide chains (in the absence of metabolite) preferentially interact with nascent polypeptide chains and block their continued synthesis through a *negative* effect.

Models 1 and 2 *may* be distinguishable in their predictions of requirements for successful *in vitro* reversal of dissociation. In model 1, the metabolite would have to be present throughout the reversal process; it could not be added after the chains had undergone a partial refolding in the absence of metabolite. In model 2, it might be possible for refolding to occur without the metabolite at all, or with metabolite added after a period of refolding in the absence of metabolite. Note that combining certain characteristics of each of the two models, other models could be obtained. One such "hybrid" model would postulate a self-inhibition of the nascent polypeptide chain which would be overcome by a *positive* stimulation of folding mediated by a completed, free polypeptide chain folded correctly for the interaction by the metabolite.

Need to Correlate Enzyme–Coenzyme Levels. It is obvious that there should be, for efficiency, a correlation between the amount of glyceraldehyde 3-phosphate dehydrogenase enzyme and its coenzyme, NAD. The stoichiometry of the correlation will be complicated by the fact that the NAD pool serves a number of dehydrogenases. If our assumptions are correct that control of folding could occur *in vivo* and be a rate-limiting step in synthesis, then NAD could control the rate of yeast glyceraldehyde 3-phosphate dehydrogenase synthesis. Furthermore, the fact that the estimated *in vivo* NAD concentration is probably less than the value 1 mM for the NAD K_F fits this idea. Since this concentration lies in the transition region of the curve for NAD enhancement of folding (Figure 8), the number of polypeptide chains folded correctly in a given period of time (and presumably the rate of synthesis) would be very sensitive to small changes in NAD concentration.

The effect produced would be reasonable from a metabolic point of view. Although it appears that the rate of synthesis would be sensitive to fairly small fluctuations in NAD levels, the total glyceraldehyde 3-phosphate dehydrogenase catalytic capacity of the cell would not change nearly so fast. Thus, this could provide for the slower, long-term adaptations to environmental circumstances, complementing the much more sensitive, short-term changes produced by control of activity of appropriate enzymes.

Control of enzyme level can involve control of rate of degradation as well as of synthesis. In this regard, it is pertinent that the coenzyme NAD protects yeast glyceraldehyde 3-phosphate dehydrogenase against a number of destabilizing effects. In particular NAD can prevent the effects of ATP, which destabilizes and dissociates yeast glyceraldehyde 3-phosphate dehydrogenase (Stancel and Deal, 1968) and markedly enhances its susceptibility to proteolytic digestion (Yang and Deal, 1969b). This could provide NAD control over degradation of the enzyme. The postulated NAD control thus has the attractive characteristic of versatility in potentially providing mechanisms for simultaneous control of both synthesis and degradation of yeast glyceraldehyde 3-phosphate dehydrogenase, with the NAD control of degradation being shared antagonistically with ATP.

A Test for Inducers and Repressors Acting by Folding Control. ANALYSIS OF METABOLITE REQUIREMENTS FOR *in vitro* REASSEMBLY. Experiments on the metabolite requirements for *in vitro* reversal of dissociation hold promise of providing a direct test whether a particular metabolite known to increase,

or decrease the rate of synthesis of a particular enzyme could be achieving this effect by influencing its rate of folding, *i.e.*, by a "folding control." Conversely, *in vitro* reassembly tests could be used to screen for potential inducers or repressors, which could operate through a "folding control" mechanism. Also, the test for enhancement of *in vitro* recovery provides an easy "screening" device to determine whether a given enzyme can exhibit metabolite-dependent folding. It avoids the necessity for the initial test of this by complicated *in vitro* cell-free protein biosynthesis experiments. Thus, correlation of results from protein biosynthesis experiments with tests of metabolite requirements for *in vitro* reversal of dissociation holds promise of providing further knowledge about the mechanism by which the rate of protein biosynthesis is increased or decreased.

Other Enzymes Dependent upon Small Molecules for Folding. The distinguishing characteristic of the yeast glyceraldehyde 3-phosphate dehydrogenase requirement for NAD for folding is that the NAD must be present from the initiation of the folding process. It cannot be added after an initial folding in the absence of NAD.

Several other proteins have been shown to strongly depend upon small molecules for folding, but in all of these systems, the small molecule is permanently bound, in contrast to the yeast glyceraldehyde 3-phosphate dehydrogenase system. Another difference is that the small molecules must be present to produce the functionally active conformation. A third difference is that their folding can be divided into two stages, the first of which does not require the prosthetic group and the second of which does. This is true for the Taka amylase–Ca^{2+} system (Takagi and Isemura, 1965, 1966; Friedmann and Epstein, 1967), the *Escherichia coli* alkaline phosphatase–Zn^{2+} system (Reynolds and Schlesinger, 1967), and the myoglobin–heme system (Harrison and Blout, 1965; Brewlow *et al.*, 1965). A number of other cases of lesser effects of small molecules upon the folding of enzymes have been reported (Chilson *et al.*, 1966; Hill and Kanerek, 1964). The phosphate effect on fumarase (Hill and Kanerek, 1964) may be only an ionic strength effect since a citrate–NaCl solvent gave significant reversal (Deal *et al.*, 1963b).

Mechanism of Enhancement. EFFECT ON FOLDING OR ON ASSOCIATION? Recognizing that the reassembly process can be arbitrarily divided into the two categories of folding and association, the question is "upon which of these processes does NAD exert its principal effect?" It might be argued that the effect of NAD is to promote association by acting as a "bridge" to hold two (or more) subunits together. The large size of NAD would serve this purpose well. But the possibility of the adenosine nucleotide binding one subunit and the nicotinamide another seems excluded by our kinetic analysis (Yang and Deal, 1969a).

The alteration of the NAD effect by varying concentrations of KCl provides an excellent basis for additional analysis of the mechanism of the NAD enhancement of reassembly (Figure 8). The ability of 0.8 M KCl to substitute for NAD suggests that NAD and KCl enhance the reassembly by the same general mechanism. Presumably, this involves promoting the folding process by neutralization of a region of high electric charge density. However, if the high salt enhancement operates by the same general mechanism as does NAD enhancement, this argues rather strongly against the "bridge" model of enhancement. That is, KCl does not possess the polyatomic structure and geometric dimensions necessary to provide such a bridge structure. This then leads to the conclusion that NAD must be operating to enhance folding.

EFFECT OF TEMPERATURE ON REFOLDING. The complete lack of recovery at 0° (Figure 6) is a striking observation. Since a temperature increase is so critical for recovery of active enzyme, it is of interest to know what state the enzyme attains in the refolding mixture during the 0° dilution preceding the temperature increase. This is currently under study.

The temperature results also raise the key question of whether the effect produced by increase in temperature is: (1) *to shift the equilibrium constant* to favor tetramers (or to favor folding) or (2) *to provide the necessary activation energy* for folding or association, or (3) both. If the temperature increase from 0 to 16° were shifting the equilibrium, then the over-all free energy of the reaction would have to be strongly temperature dependent and, in particular, favor subunits at 0°. If this were so, native enzyme should dissociate into subunits or inactive enzyme upon standing in the refolding mixture after dilution at 0°. In the temperature studies (Figure 6), the native control at 0° has a higher activity after 29 hr (300) than it does after only 120 min (270). It appears that, rather than losing activity, the enzyme is activated somewhat upon standing in the refolding mixture at 0°.

This negative evidence argues against the equilibrium being the major factor being affected. However, there is other evidence that under certain conditions, the yeast enzyme may show a temperature-dependent dissociation (G. M. Stancel and W. C. Deal, Jr., in preparation). In the case of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase the equilibrium is definitely shifted by a temperature change (Constantinides and Deal, 1967, 1968a,b; S. M. Constantinides and W. C. Deal, Jr., in preparation). This requires further study in the yeast system. The present data clearly suggests that for yeast glyceraldehyde 3-phosphate dehydrogenase one major effect of temperature is upon the activation energy for folding.

The General Applicability of the Yeast Glyceraldehyde 3-Phosphate Dehydrogenase Refolding Procedure to Other Enzymes. The question arises as to whether the conditions found are general optimum conditions for many or most enzymes, or whether the optimum conditions or reassembly are highly enzyme specific. Obviously, any metabolite effects on reassembly would be expected to be enzyme specific. So the question must be raised with respect to the general conditions which normally influence protein conformation or stability, namely, pH, temperature, protein concentration, salt species, salt concentration, reducing agent species, dissociation solvent, and procedure and refolding solvent and procedure.

Initial experiments in this laboratory on reassembly of other enzymes modeled after the yeast glyceraldehyde 3-phosphate dehydrogenase procedures described in this paper were immediately successful in yielding substantial reassembly of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (Deal and Constantinides, 1967) and rabbit muscle pyruvate kinase (Johnson *et al.*, 1969). In addition, similar success was obtained using this procedure in another laboratory with UDP-glucose pyrophosphorylase (S. Levine and R. G. Hansen in preparation). Furthermore, the conditions will also yield substantial recovery for certain other enzymes which have previously been reassembled with other procedures and conditions. These include the refolding of the rabbit muscle en-

zymes, aldolase (Deal *et al.*, 1963a,b; Stellwagen and Schachman, 1962), and α-glycerophosphate dehydrogenase (Deal *et al.*, 1963a; van Eys *et al.*, 1964; Chilson *et al.*, 1966; Holleman and Deal, 1969). We therefore conclude that these dissociation and reassembly conditions and procedures are likely to be generally satisfactory for most enzymes.

The following section summarizes the conclusions from this paper regarding the basic variables and their probable acceptable ranges.

Several key points regarding the dissociation conditions and procedures will be noted first. One problem in dissociation involving guanidine or urea is decomposition of the solvent. For this reason, it is desirable: (1) to perform the dissociation in the cold (0°), (2) to have about 0.2 M mercaptoethanol present, and (3) perhaps, to have about 0.2 M ammonium sulfate present.

For the reassembly, a key step is that the enzyme should first be diluted at least 100-fold (with well-chilled pipets) into the reassembly solvent at 0°. For some mammalian enzymes, a lengthy (30 min to 6 hr) preincubation at 0° has been found helpful (Deal and Constantinides, 1967; Johnson *et al.*, 1969). Generally though, the dilution at 0° can be followed by immediate incubation at 16°. Recovery has been obtained in the range of 12–25° for most enzymes. A pH of 6.9 using 0.05 M imidazole buffer is recommended; values between pH 6.2 and 8.5 have been found satisfactory. For optimum conditions, a protein concentration of about 0.05 mg/ml is recommended; values as low as 0.02 mg/ml have given good recovery, but in very few cases have values greater than 0.1 mg/ml given very good recovery. For ionic strength, 0.15 M KCl provides a convenient salt species and concentration; values lower than 0.05 M are generally poor but values up to 0.8 M have frequently given good recovery.

In regard to the reducing agent, 0.08 M mercaptoethanol has given results as good as glutathione and it is much more convenient to use. In some cases, glutathione specifically causes some instability, although almost always giving significant recovery. Values as low as 0.03 M aid reversal significantly, but values above 0.15 M frequently tend to inhibit reassembly somewhat for short-term (6 hr at 16°) experiments. On the other hand, for experiments involving several days exposure to 16°, concentrations of about 0.10 M are mandatory, regardless of species of reducing agent used.

In the course of the numerous reversal experiments which have been performed by other workers (see Reithel (1963) and Epstein *et al.* (1963) for reviews), some of these various conditions have been used and some have been emphasized. But in most of the cases, one or more of the recommendations given for attaining satisfactory reversal were different than those given here. Furthermore, some of these have used much more complicated reassembly procedures, *e.g.*, involving dialysis (Hill and Kanarek, 1964) and/or reconcentration of protein (Epstein *et al.*, 1964). The main advantages of the system described here are that it (1) is simple, (2) gives excellent recovery, and (3) has proven successful in tests on a number of different enzymes.

Methods

Enzyme Preparation. Yeast glyceraldehyde 3-phosphate dehydrogenase was prepared from Red Star bakers yeast, using the modification by Krebs and coworkers (Krebs *et al.*, 1953; Krebs, 1955) of the preparation of Kunitz and MacDonald (1946) for the protein designated "Yeast Protein 2." This was shown by Krebs and coworkers to be glyceraldehyde 3-phosphate dehydrogenase (Krebs *et al.*, 1953; Krebs, 1955). The enzyme was recrystallized three to four times and always tested for gross physical purity by sedimentation velocity analysis before use.

Enzyme Concentration. The protein concentration was determined from absorption measurements at 280 mμ using the approximate extinction coefficient of $E_{280\ m\mu}$ of 1.0 ml/g cm, for both the yeast glyceraldehyde 3-phosphate dehydrogenase and for other work in this laboratory on rabbit muscle glyceraldehyde 3-phosphate dehydrogenase. While this work was in progress, Kirschner *et al.* (1966) and Jaenicke *et al.* (1968) reported identical values of $E_{280\ m\mu}$ of 0.86 ml/g cm for yeast glyceraldehyde 3-phosphate dehydrogenase.

Enzyme Assay. The enzyme assay is described in some detail here since this will serve as a reference for a number of studies soon to be reported. The glyceraldehyde 3-phosphate dehydrogenase assay always showed a decrease in activity with the time of assay, due to product inhibition of the enzyme by $NADH_2$ (Krimsky and Racker, 1963), 1,3-diphosphoglyceric acid (Velick and Furfine, 1963) and due to D-glyceraldehyde 3-phosphate deterioration (S. M. Constantinides and W. C. Deal, Jr., 1967, unpublished results). Because of the latter factor, it was found to be very important that the substrate glyceraldehyde 3-phosphate be added last, since it decomposes with time. Steps were also taken to obtain accurate data in the first few seconds of the assay, before $NADH_2$ and 1,3-diphosphoglyceric acid had accumulated, or D-glyceraldehyde 3-phosphate had deteriorated; this was achieved by using an automatic high-speed recording spectrophotometer and the additional procedures described below. For the assays in the present paper, a high concentration of NAD ("high NAD–D-glyceraldehyde 3-phosphate assay") was used to neutralize better the competition of product $NADH_2$ with substrate NAD. Also our discovery (G. M. Stancel and W. C. Deal, Jr., 1967, unpublished results), that the assay was linear over a much longer period of time in the presence of salt, led to the incorporation of 0.1 M KCl into the assay.

There are two assays: (1) a "low NAD–D-glyceraldehyde 3-phosphate assay" (the standard assay) and (2) a "high NAD–D-glyceraldehyde 3-phosphate assay," in which the assay concentrations of NAD and D-glyceraldehyde 3-phosphate are increased 18-fold and 2-fold, respectively (and the pH of the stock NAD is adjusted to 6). The latter was used for all work described in this paper.

Following Krebs and coworkers (1953, 1955) the Warburg and Christian (1939) assay for glyceraldehyde 3-phosphate dehydrogenase was modified to include cysteine; the buffer was changed from pyrophosphate to glycylglycine. The standard "low NAD–D-glyceraldehyde 3-phosphate assay" used in this laboratory for yeast glyceraldehyde 3-phosphate dehydrogenase contains, in 0.4 ml, the following components (final concentrations are given in parentheses): (1) 0.24 ml of 0.1 M glycylglycine, pH 8.50 (60 mM); (2) 0.02 ml of 2 M KCl, (100 mM); (3) 0.02 ml of 0.2 M disodium arsenate (10 mM); (4) 0.05 ml of 0.06 M cysteine (H^+) (7.5 mM); (5) 0.04 ml of 0.01 M NAD (H^+) (1 mM); (6) 10 μl of enzyme solution at a concentration of 0.05 mg/ml (1.25 μg/ml); and (7) 0.02 ml of 0.01 M "stock" DL-glyceraldehyde 3-phosphate (H^+) (0.5 mM). The final pH was 7.8. Additions were made in the

order given, with mixing after steps 5–7. Mixing was by inversion using Parafilm as a cover for the microcuvets (Pyrocell No. 1009).

The "high NAD–D-glyceraldehyde 3-phosphate assay" used: (1) 0.04 ml of 0.18 M NAD stock (pH 6, final concentration 18 mM) and (2) 0.04 ml of stock D-glyceraldehyde 3-phosphate, with buffer volume reduced to 0.22 ml to compensate; the remaining components were as above.

Stock solutions of all other assay components except the glycylglycine buffer were prepared at the concentration listed above, without adjustment of pH; the pH 8.5 glycylglycine buffer stock compensated for the acidity of the added components, dropping as a result, to the desired final pH of 7.8. A several months supply of "GAK assay stock" (glycylglycine–arsenate–KCl) was prepared by mixing the appropriate volumes of these components. There were two options for further stock preparation, the choice depending upon the number of assays to be done and the time allowable per assay by the experiment design. In either case, the assay stocks were prepared just prior to beginning assays. The first additional stock option was a "cysteine–GAK" assay stock. It is stable for about 6 hr, allows NAD to be added separately, and is preferred for all work except that involving numerous assays, or kinetic analysis where assays must be run in rapid succession. The second additional stock option was an "NAD assay stock" which contained everything except the enzyme and substrate. It is stable about 2 hr.

Assays were run on a Gilford apparatus consisting of a Beckman DU monochrometer, the Gilford absorbance indicator, and a Minneapolis-Honeywell high-speed recorder. Assays were usually reproducible to within 5% and frequently better than 2%. The specific activities (micromoles per minute per milligram of protein) were calculated utilizing the extinction coefficient (Horecker and Kornberg, 1948) of 6.22 ml/μmole cm for $NADH_2$ at 340 mμ.

Preparation of Assay Components. NAD (Sigma or P-L Biochemicals) was prepared at a concentration of 0.2 M and diluted to 0.01 M for the "low NAD–D-glyceraldehyde 3-phosphate assay" or titrated with 2 M sodium hydroxide, yielding a final concentration of 0.18 M at pH 6 for the "high NAD–D-glyceraldehyde 3-phosphate assay," for refolding experiments. For the "high NAD–D-glyceraldehyde 3-phosphate assay," NAD was prepared fresh daily. The substrate D-glyceraldehyde 3-phosphate (Sigma) was prepared from the monobarium salt of the DL-glyceraldehyde 3-phosphate diethyl acetal derivative (Sigma) by adding 60 ml of water and 15 g of acid and base-washed Dowex 50 (H^+) to 1 g of the D-glyceraldehyde 3-phosphate derivative, inserting into a boiling-water bath for 3 min, and quickly chilling in an ice bath. The acid stock D-glyceraldehyde 3-phosphate solution decanted from the resin was used without dilution or titration. The product was divided into several different tubes containing 2–4 ml, covered with Parafilm, and kept frozen until used. The concentrations of the substrates NAD and D-glyceraldehyde 3-phosphate were measured spectrophotometrically using enzymatic analysis.

Dissociation Solvent and Procedure. The dissociation solvent was prepared fresh daily in 5-ml portions in a 15-ml conical tube using the following protocol: (1) 2.4 g of urea, (2) 2.18 ml of H_2O, (3) 0.5 ml of 2 M $(NH_4)_2SO_4$, (4) 0.25 ml of 0.1 M EDTA, and (5) 0.07 ml of β-mercaptoethanol (added last, after the urea has been fully dissolved). The dissociation solvent was prepared immediately prior to use, chilled in ice for several seconds, and then 1.0 ml was withdrawn and added to 5 mg of enzyme in a pellet obtained from centrifugation of an ammonium sulfate suspension of the enzyme. The enzyme was routinely dissociated at a concentration of 5 mg/ml. It was incubated in the 8 M urea dissociating medium at 0° for a minimum of 1 hr (and usually 3–5 hr) as a precaution to assure complete dissociation, although it was known that dissociation occurred within minutes of addition of the enzyme to the dissociation mixture (Deal, 1963; Deal and Holleman, 1964; W. C. Deal, Jr., in preparation). At this low temperature, standing in the urea for as long as 5 hr did not greatly decrease the degree of recovery upon reassembly, but samples aged considerably longer showed much lower activity upon reassembly, as did samples using urea solutions which were not fresh.

Refolding Solvent and Procedure. The protocol for preparation of the 1.0-ml tubes of refolding solvent, in which all variables were optimum, was the following (final concentrations are given in parentheses): (1) 0.50 ml of 0.1 M imidazole, pH 6.9 (0.05 M imidazole); (2) 0.15 ml of 0.183 M NAD, pH 6 (27 mM); (3) 0.10 ml of 0.27 M glutathione, pH 7 (27 mM); and (4) 0.15 ml of 2.0 M KCl (0.3 M); and (5) 0.10 ml of H_2O. In certain indicated experiments where a final concentration of 0.50 M KCl was used, 0.25 ml of KCl was added and water was omitted.

For the refolding 10 μl of the urea-dissociated enzyme, at a concentration of 5 mg/ml, was diluted into 1.0 ml of freshly prepared refolding solvent, giving a 100-fold dilution of the urea. It is extremely important that the refolding solvent and enzyme be near 0° during this dilution of the dissociating agent. To further ensure this condition, the refolding mixture was allowed to chill thoroughly in 15 × 100 mm test tubes in an ice bucket before adding the enzyme. For the dilution the tip of the 10-μl pipet was placed just below the surface of the refolding solvent and gently moved about in circles as the enzyme flowed from the pipet. The momentary equilibration in the 0° refolding solvent, before dilution upon shaking, helped chill the enzyme further and ensure that it was as near as 0° as possible. The tubes of diluted enzyme solution were left at 0° until enzyme had been diluted into all tubes. Then, the refolding enzyme samples were all placed in a water bath at the appropriate refolding temperature which, unless indicated otherwise, was 16°. The enzyme was allowed to remain in the refolding solvent at the refolding temperature for 1 hr or longer (up to 6 hr) and then assayed. The length of time of incubation above 60 min has very little effect upon activity, but it was kept as constant as possible for the native controls and refolding samples for a given experimental study. Other effects are described in the text.

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