The Major Anoxic Stress Response Protein p34 Is a Distinct Lactate Dehydrogenase†

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ABSTRACT: Anoxic stress is a common physiological stress, but one with unusual and significant consequences. Anoxic stress results in efficient induction of gene amplification and also plays a controlling role in the production of angiogenesis factor by macrophages. Within tumor masses, cancer cells continue to proliferate under oxygen tensions substantially lower than seen in normal tissues. The molecular basis of the anoxic stress response has not been well characterized. The major anoxic stress protein in subconfluent cell cultures is a 34-kilodalton polypeptide which has been variously reported to be either a new isozyme of lactate dehydrogenase (LDH) or the conventional muscle-type lactate dehydrogenase. This protein is of particular interest since it is also found expressed at high levels in many human cancers and has been demonstrated to be an effective serum cancer marker. We have developed an affinity chromatography procedure for purification of the anoxic stress protein p34 which effectively separates this protein from LDH-5 as well as other standard LDH isozymes. Anoxic stress protein p34 was found to specifically interact with flavins and the cellular alarmone guanosine(5′)tetraphospho(5′)guanosine, and also to interact with certain nucleic acids. The properties of this protein suggest that its overall role in the anoxic stress response may be in the coordination of a number of cellular systems.

Cellular stress response mechanisms exist which appear intimately linked to key control points in cell physiology. Two sets of different well-studied stress response proteins and genes have been identified which are induced in cells in response to heat shock and glucose deprivation (Schlessinger et al., 1982; Pelham, 1986; Munro & Pelham, 1986). In the case of heat shock, induction of the heat shock genes appears to represent a true survival response. Heat shock genes have further been linked to abnormal differentiation (phenocopy induction in *Drosophila*) (Mitchell & Lipps, 1978) and have been associated with the functioning of the *src* oncogene (Opperman et al., 1981).

Anoxia represents a third stress with limited relatedness to both heat shock and glucose deprivation. Heat shock genes are induced upon return of anoxic cells to aerobic culture conditions (Sciandra et al., 1984). The two major glucose-regulated proteins of 94 and 78 kilodaltons are produced upon anoxic stress of dense cell cultures, although this phenomenon may represent depletion of glucose in the culture media (Sciandra et al., 1984). Anoxia appears to represent a considerably milder stress than either heat shock or glucose deprivation, since cultured fibroblasts maintain full viability under anoxia for at least 3 days (Anderson et al., 1988), and unlike the other two stresses, anoxia is also associated with induction of gene amplification (Rice et al., 1986) and production of angiogenesis factors (Knighton et al., 1983).

Anoxic stress of cultured rat fibroblasts results in massive induction of VL30 element RNA, a transposon-like element (Anderson & Matovcik, 1977). This RNA induction is not seen with a variety of respiratory poisons nor with known inducers of either the heat shock genes or the glucose-regulated proteins (Anderson et al., 1988). Most of the induced RNA is polyadenylated and polysome associated, suggesting its translation results in specific anoxic stress gene products (Anderson et al., 1979).

In addition to the two glucose-regulated proteins, anoxic stress of subconfluent fibroblast cultures results in production of two polypeptides of 56 and 34 kilodaltons (Anderson et al., 1979, 1983). Previous studies in our laboratory associated these polypeptides with a lactate dehydrogenase activity, designated LDH_k[1] (Anderson et al., 1981, 1983). This activity and these two anoxic stress polypeptides were also found expressed in cells transformed by Kirsten murine sarcoma virus, a virus which contains a major fraction of a rat VL30 element as part of its genome (Anderson et al., 1988).

It has not been clear which of the anoxic stress polypeptides possesses LDH_k activity. Furthermore, it has been proposed that LDH_k might be the conventional muscle lactate dehydrogenase LDH-5 (Morin & Hance, 1983; Evans et al., 1985). To resolve these issues, we have developed an effective affinity chromatographic procedure to purify the 34-kilodalton anoxic stress protein to homogeneity. Characterization of the purified protein demonstrates it is a unique isozyme of lactate dehydrogenase, one which possesses distinct biochemical and enzymatic characteristics which indicate it has the potential to interact with a number of cellular systems.

Experimental Procedures

Cell Lines and Tissue Culture. Kirsten sarcoma virus and the Fischer rat embryo line were originally obtained from the laboratory of Stuart Aaronson.

Anoxic Stress and Two-Dimensional Gels. Fischer rat embryo cells were subjected to anoxic stress by incubation in

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[1] Abbreviations: asp34, anoxic stress protein of 33.5 kilodaltons; LDH_k, lactate dehydrogenase isozyme k; Gp_4G, guanosine(5′)tetraphospho(5′)guanosine; FRE, Fischer rat embryo fibroblast cell line; KiMSV, Kirsten murine sarcoma virus; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; PAGE, polyacrylamide gel electrophoresis; FMN, flavin mononucleotide; kDa, kilodalton(s); NBT, nitroblue tetrazolium; PMS, phenazine methosulfate.

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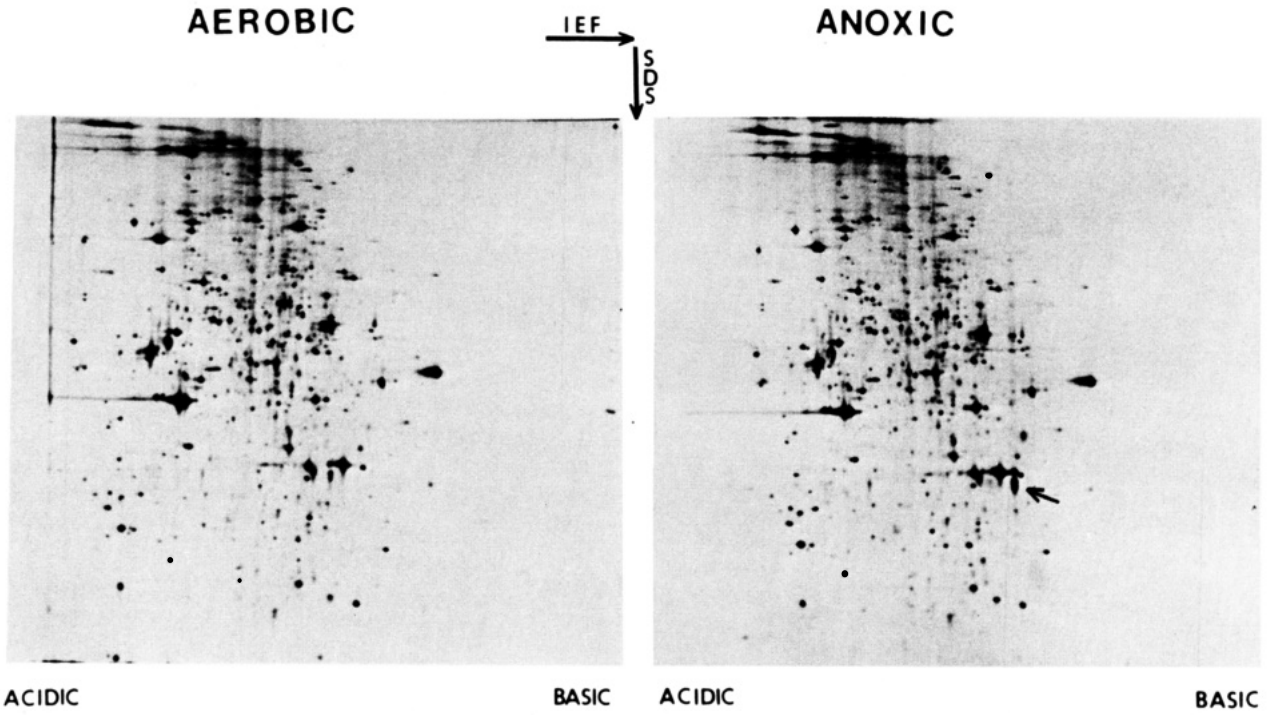

FIGURE 1: Two-dimensional nonequilibrium O'Farrell gels of anoxic stress response polypeptides. FRE cells were labeled in parallel with [^{35}S]methionine either while cultured aerobically (left panel) or while cultured from 4 to 24 h after the onset of anoxia (right panel). Labeling was done via catheter. Cells were lysed in boiling SDS buffer, treated for 1 min on ice with DNase and RNase, and frozen in liquid nitrogen. Samples were then shipped on dry ice to the Cold Spring Harbor two-dimensional gel facility, in collaboration with Mike Lambert. Nonequilibrium gels are shown, as they more effectively resolve basic proteins. The arrow designates the major LDH_k polypeptide complex at 34 kilodaltons, inducible by anoxia.

a Gas Pak anaerobic chamber. Cultures were labeled with [^{35}S]methionine (0.1 mCi/mL) which was introduced via a catheter after onset of anoxia, as determined by a glucose/methylene blue indicator. Cells were lysed in a boiling solution of 0.3% SDS, 1% β-mercaptoethanol, and 0.05 M Tris, pH 8.0. Nonequilibrium two-dimensional gels were performed in collaboration with Michael Lambert at the Quest 2-D gel lab at Cold Spring Harbor, NY.

Lactate Dehydrogenase Assay and Isozyme Gels. Total LDH assay spectrophotometrically monitors the conversion of NADH to NAD^+ in a pyruvate-utilizing reaction, or NAD^+ to NADH in a lactate-utilizing reaction.

Nondenaturing gel electrophoresis is used for LDH isozyme analysis. Two slab gel systems are used. Normal polarity gels, detecting the standard isozymes of LDH which migrate anodally, are run according to the procedure of Dietz and Lubrano. This is a 5.5% gel in a Tris/glyine buffer, with the gel at pH 8.9 and the electrophoresis buffer at pH 8.3.

Reverse polarity gels are used to assay LDH_k activity; these gels are based on an imidazole/borate buffer system. These are 5.5% polyacrylamide gels containing 0.15 M potassium borate, pH 8.3. The reservoir buffer is composed of 0.08 M imidazole and 0.02 M boric acid, pH 8.3. Gels are run at a constant voltage of 220 V, with samples migrating toward the cathode. Cooling is provided by circulating ice/water. Electrophoresis is for 16–24 h.

In all cases, at the completion of the electrophoresis, gels are stained specifically for lactate dehydrogenase activity by the nitroblue tetrazolium/phenazine methosulfate procedure of Dietz and Lubrano (1967). Gels are fixed with methanol/acetic acid and then photographed or scanned with a tungsten filament recording densitometer.

Purification of Anoxic Stress Protein p34. Tissue culture cells are homogenized in a buffer (TD) containing 0.1 M Tris-HCl, pH 8.4, and 5×10^{-4} M dithiothreitol. A 100000*g* supernatant is prepared by centrifuging 1 h at 40 000 rpm in a Ti50 rotor. The supernatant is then either used immediately or frozen at −70 °C. Chromatography is carried out on a trisacryl-blue (LKB) column, whose length is twice its diameter to achieve high flow rates. The column is washed with 20 column volumes of TD buffer. LDH isozymes 1–4 are then eluted with 10 column volumnes of 1 mM NAD^+/1 mM lithium lactate in TD buffer. LDH-5 and anoxic stress protein p34 are then eluted together in 4 column volumes of 1 mM NADH in TD buffer. The LDH-5- and asp34/LDH_k-containing pool is then immediately loaded on a column of oxamate–agarose (Sigma) in TD buffer; the column dimensions are equal to those of the trisacryl-blue column. LDH-5 (with some of the asp34/LDH_k) is then rapidly washed through the column with 20 column volumes of TD buffer plus 1 mM NADH. Following washing with 5 volumes of TD, pure asp34 is then eluted with 0.02 M lithium lactate in TD. The entire column chromatography steps are completed within 2 h, and assays of fractions are carried out only following completion of both column runs.

RESULTS

Asp34 as an Anoxic Stress Protein. One-dimensional SDS gel analysis has presented a relatively crude picture showing some of the major stable anoxic stress response proteins (Anderson et al., 1983; Sciandra et al., 1984). A much clearer picture can be obtained on two-dimensional gels, although the highly basic nature of asp34 has precluded its detection on standard O'Farrell gels (O'Farrell, 1975). Nonequilibrium two-dimensional gels performed on extracts of FRE cells subjected to long-term (20 h) labeling while under anoxia clearly revealed the high levels of asp34 induced by this stress (Figure 1).

Affinity Chromatographic Purification of Asp34/LDH_k. Cibacron-blue (trisacryl-blue, LKB) affinity chromatography

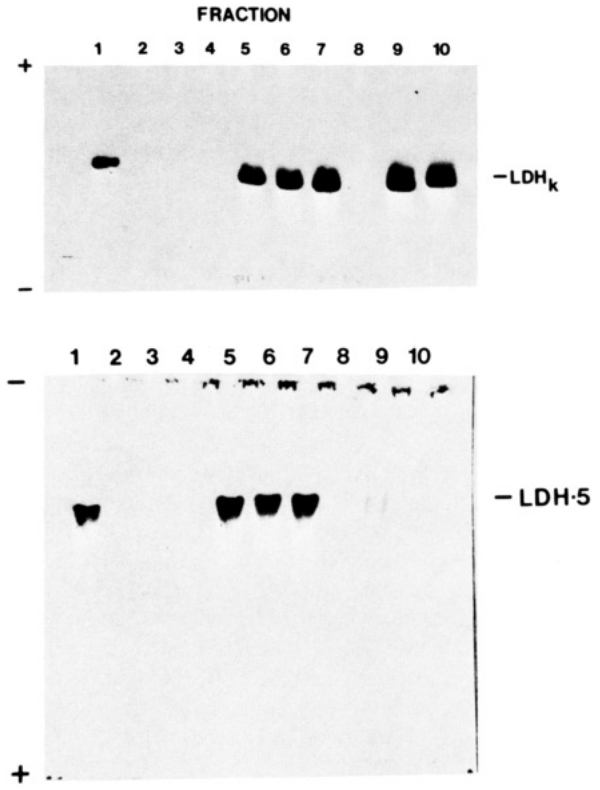

FIGURE 2: Purification of asp34/LDH$_k$ by rapid chromatography on trisacryl-blue and oxamate–agarose. A 100000*g* supernatant of an extract of Kirsten sarcoma virus transformed FRE cells was chromatographed and stepwise eluted from trisacryl-blue (fractions 1–5), fraction 5 being the NADH eluate containing asp34/LDH$_k$ and LDH-5. Fraction 5 was then loaded on oxamate–agarose and washed with TD buffer, eluting LDH-5 with some asp34/LDH$_k$ in fractions 6, 7, and 8, and then asp34/LDH$_k$ alone eluted with lithium lactate in TD buffer (fractions 9 and 10). Ten microliters of fractions 1–5 and 30 μL of fractions 6–10 were assayed for LDH isozyme activity by nondenaturing gel electrophoresis followed by activity staining. (Upper panel) LDH$_k$ activity detected by reverse polarity electrophoresis on imidazole/borate gels. Fractions 9 and 10, the oxamate eluate, show LDH$_k$ activity free of LDH-5 activity. (Lower panel) LDH-5 activity detected by normal polarity electrophoresis on Tris/glycine gels. (SDS gel analyses of these fractions are shown in Figure 3.)

has widely been used by us and others to isolate LDH-5 and/or LDH$_k$ activity away from the more acidic isozymes of lactate dehydrogenase, LDH-1 through LDH-4. This procedure uses an NADH-containing buffer to elute LDH-5 and LDH$_k$, following prior elution of the more acidic isozymes by NAD$^+$ and lactate-containing buffers. Oxamate–agarose, by serving as a substrate analogue, has also been demonstrated as an effective resin for affinity chromatographic purification of lactate dehydrogenases. In this case, lactate is used to elute LDH from the column.

Lactate dehydrogenases have differential affinities for lactate, depending on whether the lactate cosubstrate NAD$^+$ or the pyruvate cosubstrate NADH is present (Holbrook et al., 1975). We have utilized this effect to separate asp34/LDH$_k$ from LDH-5. Chromatography of a mixture of LDH-5 and asp34/LDH$_k$ on oxamate–agarose in a NADH-containing buffer results in separation of these two particular LDH isozymes (Figure 2). LDH-5 is not retained on the column, while a majority of the asp34/LDH$_k$ is retained and then subsequently eluted with lactate. A representative purification of asp34/LDH$_k$ from a 100000*g* supernatant of Kirsten sarcoma virus transformed FRE rat cells is summarized in Table I. SDS gel analyses of the oxamate–agarose column fractions make it readily apparent that LDH$_k$ has a subunit molecular

Table I: Rapid Affinity Chromatographic Purification of Asp34/LDH$_k$[a]

fraction	protein	LDH$_k$ act. (GU)	LDH-5 act. (GU)
cells: KiMSV-FRE (0.5 g)			
100000*g* supernatant	7 mg	2800	4300
trisacryl-blue	120 μg	450	780
oxamate–agarose flow through	43 μg	230	300
oxamate–agarose eluate	15 μg	190	4

[a] Column purification is detailed under Experimental Procedures. LDH$_k$ and LDH-5 activities were determined by activity staining of nondenaturing gels and scanning densitometry, where a GU is one integrating densitometer unit.

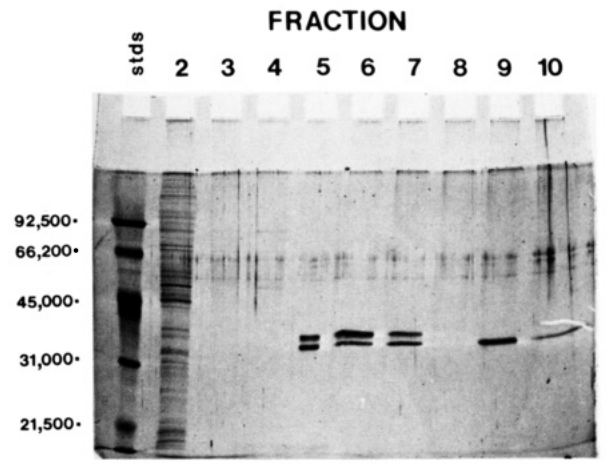

FIGURE 3: SDS–polyacrylamide gel analysis of asp34/LDH$_k$ isolated by the rapid affinity chromatography procedure described in Figure 2. 10% SDS gels were performed on the column fractions of Figure 2. Bio-Rad molecular weight standards are in the left lane; proteins were visualized by silver staining. Lane 2, 2 μL of column fraction 2; lanes 3–5, 10 μL of fractions 3–5, respectively; lanes 6–10, 30 μL of fractions 6–10, respectively. Fractions 5, 6, and 7, which have both asp34/LDH$_k$ and LDH-5, show a clear doublet, while fractions 9 and 10, with asp34/LDH$_k$ alone, show only the lower band.

weight of about 33 500, approximately 1K smaller than that of LDH-5 (Figure 3). It should be noted that the retained oxamate–agarose peak contains material which assays only as LDH$_k$ but not as LDH-5. A critical requirement is that the chromatography steps must be carried out rapidly, with the total purification occurring typically in less than 4 h. If the oxamate–agarose column step is delayed, LDH-5 also is retained on the oxamate–agarose column and contaminates the asp34/LDH$_k$ fractions.

This affinity chromatography procedure also has been effective in isolating asp34/LDH$_k$ from extracts of tissues heavily expressing the LDH-H subunit, as is often the case in human tumors. In such cases, the three different subunits, asp34/LDH$_k$, LDH-M, and LDH-H, are all resolved with different molecular weights on SDS gels followed by silver staining.

Asp34/LDH$_k$ could represent posttranslationally modified or proteolytically clipped LDH-5; alternatively, asp34 could represent a totally distinct gene product. We therefore compared the V-8 protease generated peptides of asp34/LDH$_k$ with those from LDH-5, using these polypeptides metabolically labeled with [^{35}S]methionine. Fifteen 10-cm culture dishes of Kirsten sarcoma virus transformed FRE rat cells were labeled for 4 h with 5 mCi of [^{35}S]methionine, and the asp34 was isolated as described above. In parallel, the oxamate–agarose flow-through fraction provided a source of highly purified asp34 and LDH-5 together. When compared by V-8 proteolysis followed by SDS–PAGE and autoradiography, it was clear that the asp34/LDH$_k$ + LDH-5 pool contained all

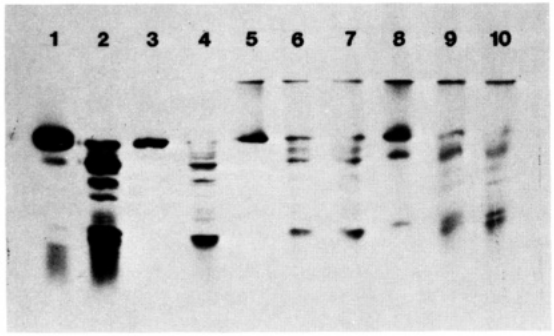

FIGURE 4: Cleveland gel analysis of [^{35}S]methionine-labeled asp34/LDH$_k$ and LDH-5. Kirsten sarcoma virus transformed FRE cells were metabolically labeled with [^{35}S]methionine for 4 h, followed by purification of the asp34/LDH$_k$ by the rapid chromatography procedure. V-8 digestion was for 1 h at 37 °C in Cleveland gel, and 15% SDS–polyacrylamide gels were used for peptide analysis. Lane 1, asp34/LDH$_k$ with LDH-5 (oxamate column flow through, approximately equal asp34/LDH$_k$ and LDH-5, ca. 5 μg of protein); lane 2, lane 1 material plus 2 μg of V-8 protease; lane 3, asp34/LDH$_k$ alone; lane 4, lane 3 material plus 2 μg of V-8 protease; lane 5, lane 3 material, preelectrophoresed on an imidazole/borate gel and the material banding with LDH$_k$ activity was then loaded here; lane 6, lane 5 material plus 1 μg of V-8 protease; lane 7, lane 5 material plus 2 μg of V-8 protease; lane 8, lane 1 material, preelectrophoresed on a Tris/glycine gel and the material at the position of LDH-5 was then loaded here; lane 9, lane 8 material plus 1 μg of V-8 protease; lane 10, lane 8 material plus 2 μg of V-8 protease.

those peptides present in asp34/LDH$_k$ alone, along with a distinct second set corresponding to LDH-5 (Figure 4). To ensure that the observed V-8 peptides indeed corresponded to asp34 or LDH-5, the same [^{35}S]methionine-labeled fractions were also preelectrophoresed on imidazole/borate- or Tris/glycine-buffered gels, and the material migrating at the positions of asp34/LDH$_k$ or LDH-5, respectively, was then subjected to V-8 digestion (Figure 4). The material electrophoresing as asp34/LDH$_k$ again showed a pattern quite distinct from the LDH-5 material but indicated both asp34/LDH$_k$ and LDH-5 were present in the Tris/glycine-buffered gels. This in fact was later confirmed (see below). Consistent results were obtained when unlabeled asp34/LDH$_k$ isolated from Kirsten virus transformed FRE cells was compared with LDH-5 isolated from uninfected FRE cells, with the V-8 peptides visualized by silver staining. Together, these results indicate that either asp34 and LDH-5 are encoded by different genes or major posttranslational modifications are involved. Gene cloning experiments are in progress to differentiate these possibilities.

Regulation of LDH$_k$ Activity by Guanine Nucleotides. One of the most interesting properties of the LDH$_k$ activity seen with asp34 is its inhibition by two cellular "alarmones", adenosine(5′)tetraphospho(5′)adenosine (Ap_4A) and guanosine(5′)tetraphospho(5′)guanosine (Gp_4G). The alarmone Ap_4A is associated with oxidative stress in a variety of systems (Lee et al., 1983; Bochner et al., 1984), while the role, if any, for Gp_4G has yet to be precisely defined. Using cuvette assays of our highly purified asp34/LDH$_k$ and of LDH-5, we have confirmed that the sensitivity of dipurine nucleoside tetraphosphates is specific to LDH$_k$ activity (Figure 5A). We have also analyzed the effects of other diguanosine polyphosphates on LDH$_k$ activity. Only Gp_4G, but not Gp_3G or Gp_5G, inhibited LDH$_k$ activity (Figure 5B).

Interaction of Asp34/LDH$_k$ with Flavins. Cytochrome–LDH of yeast is associated with the switching of yeast from anaerobic to aerobic culture conditions and has been postulated to be a prototype of mammalian LDH activity (Anderson & Kovacik, 1981; Anderson et al., 1983). Since yeast cytochrome–LDH is known to utilize an FMN cofactor, we examined the effects of flavin compounds on LDH$_k$ activity. Relatively high concentrations of FAD are inhibitory of LDH$_k$ activity. This effect is not observed with two other isozymes of LDH which we tested, LDH-1 and LDH-5. The K_i for FAD is approximately 1×10^{-4} M for LDH$_k$ and greater than 10^{-3} M for the other LDH isozymes.

FMN can also bind to asp34/LDH$_k$, but without apparent loss of LDH$_k$ enzymatic activity. When aliquots of asp34/LDH$_k$ were incubated with FMN, its electrophoretic mobility was shifted such that it migrated less cathodally. The K_m for such binding was 3×10^{-5} M. Binding was inhibited by

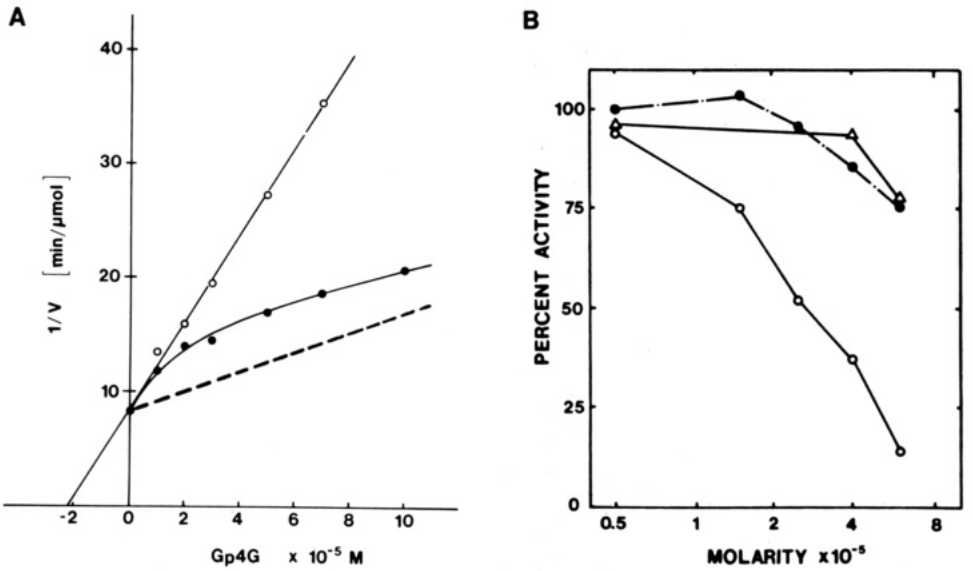

FIGURE 5: Effects of guanosine(5′)tetraphospho(5′)guanosine on LDH$_k$ activity. (A) Cuvette assays of asp34/LDH$_k$ for the reactions lactate → pyruvate and NAD^+ → NADH were carried out in the presence of the indicated amounts of Gp_4G, in a phenazine–nitroblue tetrazolium coupled assay. Activity was determined by measuring A_{620}. Samples were 0.1 μg of purified rat LDH$_k$ (○) and 0.3 μg of Sigma human LDH-5 (●). Also shown is the slight effect of Gp_4G on the chemical reaction NADH + PMS + NBT → formazan blue (- - -). One-milliliter assay mixtures containing 10 or 50 nmol of NADH, and Gp_4G at the indicated concentrations were incubated 3 min at 20 °C, and formazan blue was quantitated by measuring the A_{620}. (B) Gp_4G specificity for inhibition of LDH$_k$ activity. Asp34/LDH$_k$ purified by affinity chromatography from Kirsten-transformed FRE cells was assayed in cuvettes. Gp_3G (●), Gp_4G (○), and Gp_5G (△) were assayed at the indicated concentrations and were purchased from Sigma.

Table II: Nucleic Acid Binding by Asp34/LDH$_k$[a]

	free enzyme/total
asp34, control	1.00
asp34 + nucleic acids (100 μg/mL)	
poly(A)	0.00
poly(C)	1.10
poly(AC)	0.00
poly(G)	0.61
poly(U)	0.01
poly(I)	0.00
poly(A)/poly(U)	0.00
tRNA	0.81
asp34 + poly(dA)	0.71
poly(dT)	0.00
salmon sperm DNA	0.14
boiled salmon sperm DNA	0.01

[a] Four micrograms of enzyme was incubated with nucleic acid as indicated, for 10 min at 4 °C, in a reaction volume of 40 μL. This was then electrophoresed on an imidazole/borate-buffered nondenaturing gel, followed by activity staining for free remaining LDH$_k$ activity or by silver staining for unbound protein. Gels were then scanned on an integrating densitometer. Data shown were computed from activity measurements.

NADH, showing competitive kinetics. In parallel studies, no such FMN interaction was seen with LDH-5.

Interactions of Asp34 with Nucleic Acids. LDH-5 has been reported capable of binding to single-stranded DNA and has tentatively been identified as a hexlix-destabilizing protein (Calissano et al., 1985; Cattaneo et al., 1985). To determine if asp34/LDH$_k$ shows a similar interaction, aliquots of the purified protein were incubated with a variety of nucleic acids. Unbound enzyme was then measured following electrophoretic separation from bound complexes. As listed in Table II, asp34/LDH$_k$ bound to certain nucleic acids, but not others. No general pattern as to DNA/RNA single stranded/double stranded was apparent, although some sequence specificity evidently existed with a preference for A- and T-containing nucleic acids. Reduced NADH showed the ability to dissociate asp34 from nucleic acid. NAD$^+$ at concentrations as high as 400 μM showed no effect, while NADH showed an apparent K_i of approximately 100 μM.

Asp34/LDH$_k$ Is Not a Phosphorylated Form of LDH-5. Hunter and Cooper have shown that LDH-5 is one of three glycolytic enzymes phosphorylated substantially following transformation (Cooper et al., 1983, 1984). Although phosphorylated LDH-5 shows an isoelectric point approximately 1 pH unit lower than LDH-5, conceivably electrophoretic anomalies might cause phosphorylated LDH-5 to appear more basic in our assay system. Accordingly, we examined the possibility that asp34/LDH$_k$ represented phosphorylated LDH-5. Kirsten sarcoma virus transformed FRE cells were labeled in vivo with $^{32}PO_4$, and the asp34/LDH$_k$ was isolated by the rapid affinity procedure described in Figure 2. Nondenaturing electrophoresis gels were then performed on the fractions containing these two isozymes. Gels were activity stained for LDH and then autoradiographed for $^{32}PO_4$. As described in Table III, no $^{32}PO_4$ was found associated with asp34/LDH$_k$, at least 10-fold less than that readily observed with LDH-5. In addition, when oxamate–agarose flow-through fractions containing both asp34 and LDH-5 were analzyed on SDS gels and the bands identified with Coomassie blue staining and autoradiography, ^{32}P was found only associated with the upper band corresponding to the LDH-M subunit of LDH-5.

Assay Specificities of Gel Electrophoresis Methods. The fact that LDH$_k$ has a lesser molecular weight than LDH-5 permitted us to independently identify asp34/LDH$_k$ and

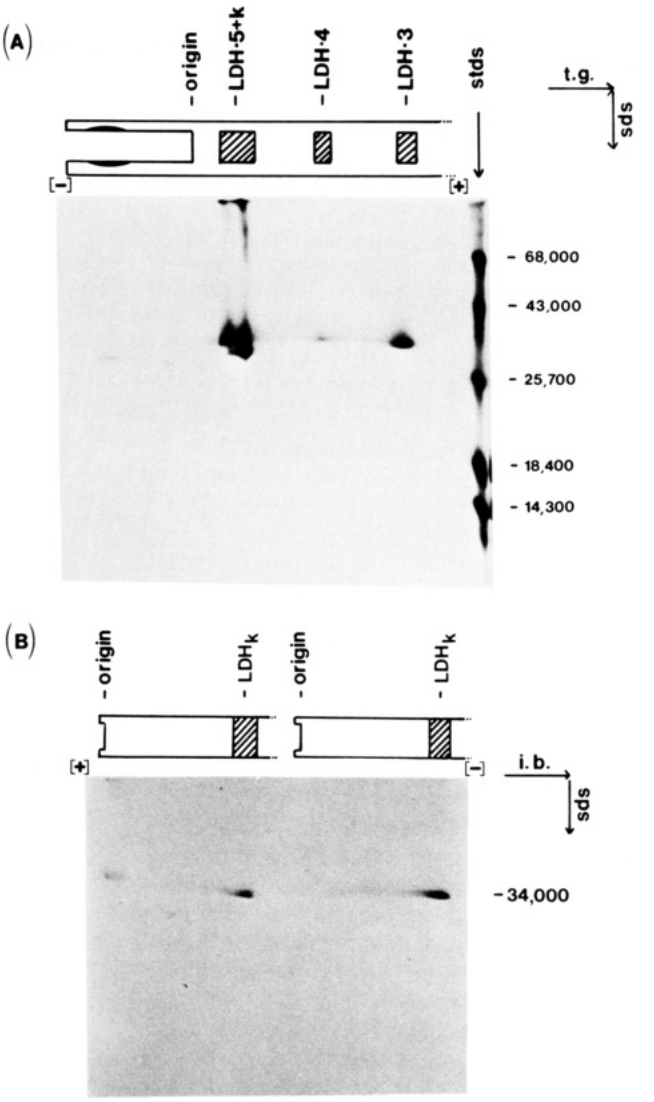

FIGURE 6: Two-dimensional gels of [^{35}S]methionine-labeled LDH, where the first dimension is an LDH isozyme assay gel. (A) The first dimension was a Tris/glycine gel system, the standard means to assay conventional LDH isozymes. Fractions containing [^{35}S]-methionine-labeled asp34/LDH$_k$ and LDH-5 were combined with a small amount of [^{35}S]methionine-labeled LDH-3 as a marker. This was electrophoresed anodally into a Tris/glycine gel. A parallel lane was stained for LDH activity. SDS gel electrophoresis was then used as a second dimension. (B) [^{35}S]methionine-labeled asp34/LDH$_k$ alone (right lane) or with LDH-5 (left lane) was first electrophoresed on imidazole/borate gels, and then as a second dimension electrophoresed onto SDS gels.

LDH-5 on Tris/glycine and imidazole/borate gels, by using SDS–PAGE (with autoradiography) as a second electrophoresis dimension. Two-dimensional gels were compared with LDH activity-stained first-dimension gels. Using these methods in studies of [^{35}S]methionine metabolically labeled purified proteins, we found that LDH-5 barely entered the imidazole/borate-buffered gels and was readily resolved from the much faster cathodally migrating asp34/LDH$_k$ (Figure 6B). In contrast, both asp34/LDH$_k$ and LDH-5 when mixed together enter conventional Tris/glycine gels and migrate close to one another (Figure 6A). When asp34 was purified free of LDH-5, however, very little of the protein entered the Tris/glycine gel.

Discussion

Over 20 years ago, Kaplan and co-workers observed that anaerobic conditions resulted in increased LDH activity with

Table III: Relative Phosphorylation of LDH-5 and Asp34/LDH_k[a]

	LDH-5	asp34/LDH_k
enzyme activity	3.5	4.0
$^{32}PO_4$ incorporation	37.8	0.0
PO_4/activity	10.0	0.0

[a] KiMSV-transformed FRE cells were labeled in vivo for 4 h with 250 μCi/mL $^{32}PO_4$. Cells were homogenized in 0.01 M Tris, pH 8.4, and 5×10^{-4} M DTT. The supernatant of a 10 min × 10000*g* centrifugation was sequentially chromatographed on minicolumns of tris-acryl-blue and oxamate–agarose to isolate lactate dehydrogenase. These were then analyzed by electrophoresis, activity staining, and scanning on an integrating densitometer. The same gels were then autoradiographed. The autoradiograms were also scanned on the integrating densitometer. In all cases, the units shown are integrating densitometer units.

kinetic properties somewhat resembling muscle-type LDH (Goodfriend et al., 1966). Our results show this anoxic stress response protein is not LDH-5 itself, but a distinct isozyme of lactate dehydrogenase with properties quite different from the conventional lactate dehydrogenase LDH-5.

Anoxic stress evidently represents a third stress system which is independent of, yet connected to, the two well-studied stress systems heat shock and glucose deprivation. Anoxic stress does not directly induce the heat shock response, but anoxic cells following return to aerobic culture induce heat shock genes. Anoxic stress also induces two major glucose-regulated proteins of 97 and 79 kilodaltons, but glucose deprivation does not induce the major anoxic stress protein asp34. (Anderson et al., 1979, 1988; Sciandra et al., 1984). Further evidence for separate regulation of glucose-regulated proteins and asp34 comes from the inability of anoxia to induce asp34 in confluent cell cultures, where grp97 and grp76 are induced at greatest levels by the same stress.

Our results make clear that limitations exist for LDH isozyme analysis by the conventional Tris/glycine gel and similarly based electrophoresis systems. Such procedures indeed detect LDH-5 but also detect LDH_k migrating to a position near that of LDH-5. The imidazole/borate-buffered gel system used for LDH_k is more useful in that only LDH_k is detected but is somewhat limited by the ability of nucleic acids to prevent measurement of LDH_k. This limitation can be overcome by adding NADH to samples being assayed.

The association of 56-, 34-, and 21-kilodalton polypeptides with impure preparations of LDH_k activity led us to suggest that its native form might have a 56-kDa subunit which is posttranslationally processed (Anderson et al., 1983). The procedures used in this report yield LDH_k with only the 34-kDa polypeptide, and long-term labeling in vivo shows the major stable LDH_k polypeptide is of this size.

Our studies confirm that anoxic stress protein p34/LDH_k is a distinct isozyme of lactate dehydrogenase. This major anoxic stress response protein has the potential of interacting with a number of systems involving carbohydrate metabolism, NADH oxidation, guanine nucleotides, and nucleic acids. This potential ability to coordinate a number of systems may reflect its true role as a stress response protein. LDH_k activity has been found at high levels in a variety of human cancers and has additionally been demonstrated to be an effective serum cancer marker (Manly et al., 1987). Not surprisingly, expression of serum LDH_k is not coordinate with LDH-5 (Petrelli et al., 1988). One role for asp34/LDH_k in cancer cells may be to permit growth under the conditions of low oxygen tension typically seen within tumors. However, the expression of this same protein in oxygenated tumors such as leukemias suggests that its putative coordination function might reflect its major role in cancer cells.

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Action of the Active Metabolites of Tiazofurin and Ribavirin on Purified IMP Dehydrogenase†

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ABSTRACT: The inhibitory mechanisms of ribavirin 5′-monophosphate (RMP) and thiazole-4-carboxamide adenine dinucleotide (TAD), the active forms of the antimetabolites ribavirin and tiazofurin, were investigated in IMP dehydrogenase purified to homogeneity from rat hepatoma 3924A. The hepatoma IMP dehydrogenase has a tetrameric structure with a subunit molecular weight of 60000. For the substrates IMP and NAD^+, K_m's were 23 and 65 μM, respectively. Product-inhibition patterns showed an ordered Bi-Bi mechanism for the enzyme reaction where IMP binds to the enzyme first, followed by NAD^+; NADH dissociates from the ternary complex first and then XMP is released. XMP interacts with the free enzyme and competes for the ligand site with IMP, while NADH binds to the enzyme·XMP complex. RMP exerted the same inhibitory mechanisms as XMP, and the inhibition by TAD was similar to that by NADH. However, the K_i values for RMP (0.8 μM) and TAD (0.13 μM) were orders of magnitude lower than those of XMP (136 μM) and NADH (210 μM). Thus, the drugs interact with IMP dehydrogenase with higher affinities than the natural substrates and products, RMP with the IMP–XMP site and TAD with the NADH site. Preincubation of the purified enzyme with RMP enhanced its inhibitory effect in a time-dependent manner. The enzyme was protected from this inactivation by IMP or XMP. These results provide a biochemical basis for combination chemotherapy with tiazofurin and ribavirin targeted against the two different ligand sites of IMP dehydrogenase.

IMP dehydrogenase (EC 1.1.1.205), the rate-limiting enzyme of de novo GTP biosynthesis, is a promising target in cancer chemotherapy (Weber et al., 1976; Weber, 1983; Robins, 1982; Tricot et al., 1987). The antimetabolites such as tiazofurin and SM-108,[1] targeted against IMP dehydrogenase, have a broad spectrum of action against tumors including Lewis lung carcinoma, which is refractory to most drugs (Yoshida et al., 1980; Robins et al., 1982). The oncolytic action of these drugs has been linked with the depletion of GTP and dGTP pools (Weber et al., 1984; Lui et al., 1984; Fukui et al., 1986).

Because of the strategic importance of the guanylate pathway and its rate-limiting enzyme, IMP dehydrogenase, it is vital to clarify the inhibitory mechanisms and the sites of drug actions. Combinations of the drugs against IMP dehydrogenase that act at different ligand sites might potentiate the inhibitory effect on guanylate synthesis. The resistance to an inhibitor might be overcome by a suitable choice of drugs with different metabolism and mechanisms. We recently purified IMP dehydrogenase to homogeneity from rapidly growing hepatoma 3924A (Ikegami et al., 1987). The present study determined the kinetic properties of purified IMP dehydrogenase with the natural ligands (IMP, XMP, NAD, and NADH) and elucidated the inhibitory mechanisms of the active metabolite of ribavirin (RMP) and tiazofurin (TAD).

Materials and Methods

Materials. [8-^{14}C]IMP was purchased from Amersham. Epoxy-activated Sepharose 6B was from Sigma and hydroxylapatite (Bio-Gel HTP) was from Bio-Rad. Sephacryl S-400 gel and an electrophoresis calibration kit were from Pharmacia. TAD and RMP were kindly supplied by Dr. R. K. Robins, Nucleic Acid Research Institute, Costa Mesa, CA. All other chemicals were of the highest purity available.

Enzyme Assay. In the enzyme purification IMP dehydrogenase activity was determined by a radiochemical assay and spectrophotometric assays (Ikegami et al., 1985, 1987). In the kinetic studies with the purified enzyme, however, adenosine 5′-α,β-methylenediphosphate and allopurinol were omitted from the reaction mixture.

Enzyme Purification. IMP dehydrogenase was purified from transplantable rat hepatoma 3924A as described by Ikegami et al. (1987) except that Sephacryl S-400 gel filtration was added to the purification steps prior to the IMP Sepharose affinity column. The gel (2.5 cm × 60 cm) was preequilibrated with 40 mM Tris-HCl, pH 7.4, containing 1 mM DTT, 1 mM EDTA, 10% (v/v) glycerol, and 0.15 M KCl. Fractions with enzyme activity were collected and applied on an IMP Sepharose column (0.8 cm^2 × 18 cm) equilibrated with the same

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[1] Abbreviations: SM-108, 4-carbamoylimidazolium 5-oleate; RMP, ribavirin 5′-monophosphate; TAD, thiazole-4-carboxamide adenine dinucleotide; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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